Ukraine Biological Threat Reduction Program (BTRP)
Cooperative Biological Research (CBR) Project

The spread of African swine fever virus (ASFV) in domestic pigs and wild boar in Ukraine – Building capacity for insight into the transmission of ASFV through characterization of virus isolates by genome sequencing and phylogenetic analysis

UP-9 PROJECT OPTION YEAR 1 FINAL REPORT
for the period 01 April 2019 – 30 June 2020

Prepared for:



Prepared by:

BLACK & VEATCH SPECIAL PROJECTS CORP.



in collaboration with:





15 July 2020

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1. Project Information

1.1. Task Order 04 information

1.1.1. Contract Number

HDTRA1-08-D-0007-0004

1.1.2. Project Title

Biological Threat Reduction Program (BTRP)

Cooperative Biological Engagement Program (CBEP)

Phase IIb in Ukraine – HDTRA1-08-D-0007-0004

1.2. CBR information

1.2.1. Project number: UP-9 Option Year 1 (OY1)

1.2.2. Project title: The spread of African swine fever virus (ASFV) in domestic pigs and wild boar in Ukraine – Building capacity for insight into the transmission of ASFV through characterization of virus isolates by genome sequencing and phylogenetic analysis

1.2.3. Performance Period

Project Period of Performance – 01 April 2019 – 30 June 2020 (OY1 and no-cost extension)

1.2.4. Performing Organization

Black & Veatch Special Projects Corp. (BVSPC)

1.2.5. Teaming Partner

Metabiota, Inc.

1.3. Threat Reduction

1.3.1. Project Impacts

Through the following accomplishments, UP-9 OY1 advanced Ukraine's scientific and technical capacity to deploy state-of-the-art genomic-based biosurveillance for African swine fever (ASF) to promote ASF detection, response, and control in the country:

- Updated an in-depth surveillance to understand spatial and genomic dynamics of ASF spread in domestic swine and wild boar in Ukraine.
- Identified internal and cross-border origins of virulent ASF virus (ASFV) genotype ASFV Georgia/2007 lineage outbreaks in Ukraine.
- Coordinated a regional collaboration and sharing of genomics-based methods for biosurveillance and control of ASF, in Ukraine, Poland, Georgia, Armenia, Moldova, and other regional partners.
- Built technical capacity for analyzing ASF, swine co-infections, and other Especially Dangerous Pathogens (EDPs), including characterization and pathogen genome sequencing by Ukrainian scientists.

- Built technical capacity for computational (bioinformatics) analysis of ASFV sequences for epidemiological mapping and robust, accurate genomics-based ASFV strain identification.
- Integrated Ukrainian scientists and Institutes into the international scientific community with adoption of international diagnostic standards, publication and dissemination of results at international conferences and in scientific publications, and collaboration with US-based, international, and regional country scientists.

1.3.2. Future Recommendations

- Integrate UP-9-derived ASF genotyping and phylogenetics-based risk maps into ongoing and future biosurveillance and disease threat reduction networks in Ukraine and with regional partners.
- Build upon expertise in Ukrainian institutes, as well as through regional and international collaborations, to foster junior scientists in nanopore (MinION) sequencing, phylogenetics, and GIS mapping methods for understanding threats posed by ASF and other pathogens.
- Strengthen mechanisms and communication for preparedness and response in Ukraine and the region by publishing and using UP-9 project data to inform public officials and scientists, expanding the ability to identify and contain regional ASF outbreaks.
- Conduct technical analyses and pursue publication of ASF epidemiological factors and genomics data on ASFV and co-infections.
- Perform phylogenetics analyses of ASFV and co-infections, as well as analysis of antigenic structures, and inform new approaches for creating vaccines to control ASF.
- Conduct epidemiological and metagenomics studies of wild boar and domestic swine to understand host susceptibility and persistence of ASFV and co-infections.
- Leverage identification of co-infections (such as PCV2) as biomarkers for understanding ASF spread and introduction by anthropomorphic means, including pork production and food supply.

2. UP-9 Project Description

2.1. Project Title

Ukraine UP-9 Quarterly Factsheet Information

"The spread of African swine fever virus (ASFV) in domestic pigs and wild boar in Ukraine – Building capacity for insight into the transmission of ASFV through characterization of virus isolates by genome sequencing and phylogenetic analysis"

2.2. Research Objectives

2.2.1. Problem Description

ASF is a serious viral disease of swine, characterized by high mortality and significant economic losses. The disease spread rapidly in Eastern Europe in 2007-2019, starting in the Caucasus from Georgia, Azerbaijan, and Armenia, then crossing the southern European region of the Russian Federation into Ukraine and Belarus (2012 and 2013), Poland and the Baltic States (2014), Romania (2017), Czech Republic (2017), and Hungary (2018). As the disease spread across Ukraine from 2012-2018, there were a total of 465 laboratory-confirmed ASF outbreaks in the country. Today, Ukraine remains in the geographic center of the ASF zoonosis and is under threat of long-term endemic disease.

The threat of ASF becoming an endemic disease or being reintroduced via transboundary ASF transmission from countries in Eastern Europe and the Caucasus poses tremendous risk to commercial and backyard swine operations in Ukraine. The ongoing spread of the etiological agent of ASF, a virulent lineage of ASFV that emerged in Georgia in 2007 and spread throughout Eastern Europe, highlights the need to identify and characterize the ASFV genotypes circulating in Ukraine. Current knowledge gaps include limited understanding of susceptibility and transmission patterns, the role of domestic pigs and wild boar in the ASFV transmission cycle, the relative virulence of circulating isolates, and the potential role of carrier animals and the pork industry. The epidemiology and evolution of ASFV, including the source and location of virus introduction for individual outbreaks, the rate of spread within the country, and the rate of evolutionary change, are poorly understood. Importantly, there is no effective vaccine nor are there antiviral drugs available for the prevention or treatment of ASFV, and control measures have been difficult to implement.

Research conducted during the UP-9 base period was key to addressing the above knowledge gaps concerning ASF spread and virulence in Ukraine. The UP-9 team developed ASFV genome sequencing expertise, resources, and capacity centered at The State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE), which maintains samples of extracted DNA from a large number of ASF outbreaks in the country. Through these efforts, the partial sequence of an isolate from a wild boar (in Luhansk) was determined and submitted to the National Center for Biotechnology Information (NCBI) GenBank (Gallardo, Fernandez-Pinero et al. 2014) (GenBank Accession no. 612065690). Additional studies, which examined short diagnostic DNA sequences of ASFV isolates from Ukraine (p72 encoded by gene B646L and the Central Variable Region [CVR] in gene B602L), confirmed that ASFV strains circulating in Ukraine are derived from the virulent ASFV Georgia/2007 lineage (Valdivia, W., TAP-6 project; SSRILDVSE, unpublished data).

Within the base period, the UP-9 team sequenced three full ASFV genomes and nine partial ASFV genomic signatures (PCR amplicons that span specific loci in the virus genome) using long-read nanopore technology. This success can be attributed to the team's development of the first deployable genotyping approach for ASFV in Ukraine using Oxford Nanopore Technologies (ONT) MinION© portable deep sequencing platform. Importantly, the full genome of ASFV circulating in Ukraine was sequenced using this approach. Additionally, laboratory and bioinformatics expertise were developed to engage in a comprehensive phylogenetic analysis of Ukraine ASFV isolates and to build similar capacity in regional partner laboratories.

Virulent ASF continues to persist and spread in Ukraine despite outbreak surveillance, evidence-based control measures, and growing awareness among state veterinary services, large commercial domestic pig producers, backyard farmers holding swine, wild boar hunters, and the public. Similar introduction and persistence of ASF has been described in Poland, the Baltics, and countries in the Eastern European Union (Nurmoja, 2017; Olesen, 2018 [a]), Russia (Malogolovkin, 2012; Kolbasov, 2018), and China (Ge, 2018). In these contexts, as in Ukraine, the ASFV/Georgia/2007 (p72 genotype II) lineage of virus has been responsible for the spread into new regions, and infections in both domestic pigs and wild boar have been observed. Despite considerable research, disease surveillance, and control efforts, it is apparent that the basic epidemiological nature of ASF spread is poorly understood and thereby challenging to mitigate.

In UP-9 OY1 collaborators developed new methodology and furthered a collaborative knowledge base for analysis of ASF spread, which contributed to understanding the molecular nature of ASFV genotypes and virulence. These advances can be leveraged as a resource for development of prevention and control measures in Ukraine, and other regions, to reduce the threat of ASF outbreaks. Collectively, the findings of the UP-9 base period and OY1 contributed to the region's preparedness and can potentially aid in multinational efforts to stem the spread of ASF.

2.2.2. Research Goals

The UP-9 project aimed to comprehensively study genomes of ASFV associated with outbreaks in Ukraine (2012-2020) and to understand the epidemiological patterns of these outbreaks. The overall goal of the project (base period and OY1) focused on investigating the genetic diversity, distribution, and spread of ASFV in Eastern Europe, with emphasis on Ukraine, as well as on informing transboundary forecasting and control strategies to reduce the risk of ASF outbreaks.

The UP-9 Final Report describes highlights of OY1 project implementation, which encompassed the following **Aims** and **Tasks**:

Aim 1. Understand genotypes of ASFV in Ukraine and track the spread of virus by genotypic signatures.

- Task 1.1. Trace the origin and spread of ASF outbreaks in Ukraine (2014-2020) by genomic signature and full genome sequencing analyses of ASFV using nanopore sequencing (ONT MinION) technology.
- Task 1.2. Understand ASFV virulence in wild boar and domestic pigs by genomics and evolutionary analysis of ASFV pathogenicity genes and detection of co-infection in swine.

Aim 2. Undertake investigations into the epidemiology of ASF in Ukraine to understand exposure, incidence, and prevalence for mapping the disease.

- Task 2.1. Estimate the roles of environmental risk factors on incidence, persistence, and geographic distribution of ASF outbreaks.
- Task 2.2. Understand ASFV exposure and prevalence in ASF outbreak zones by serological surveillance of domestic pigs and wild boar.

Aim 3. Scientific Advancement: Bioinformatics capacity-building and data sharing.

- Task 3.1. Advance scientific capacity for pathogen genomic analysis through bioinformatics and sequence analysis workshops.
- Task 3.2. Advance ASF data sharing, reporting, and international collaboration through workshops, communication among institutes in Ukraine and Subject Matter Experts (SMEs), and scientific presentations and publications.

2.2.3. Expected Impact

UP-9 OY1's focus on understanding the virus genotypes and epidemiology of ASF infections from recent outbreaks in Ukraine (2012-2020) enhanced the capacity for regional sequencing and control of ASF. Importantly, the following was accomplished:

- Genotyped the virulent ASFV/Georgia/2007 lineage strain variants circulating in Ukraine.
- Compared genotypes in Ukraine with circulating ASFV in the region, including Poland, the Baltics, and Georgia, to uncover regional outbreak dynamics.
- Advanced the scientific knowledge base and technological capacity for virus sequencing in Ukraine through partnerships with regional laboratories by employing an affordable, state-of-the-art nanopore sequencing platform (ONT MinION) and building workflows for bioinformatics analysis.

- Advanced understanding of epidemiological factors governing incidence, persistence, and virulence of ASF outbreaks in domestic pigs and wild boar in Ukraine.
- Released data on ASFV genomes to NCBI GenBank for use in regional epidemic tracing and phylogenetics analyses.
- Communicated findings to institutional and state laboratory stakeholders in Ukraine to improve implementation of ASF control measures.
- Provided mentorship and international collaborative opportunities for scientists in Ukraine.
- Created partnerships in the region for presentation and peer-reviewed publication of research.

Through study of ASFV genotype variation and ASF outbreak mapping, UP-9 OY1 activities have contributed to expanding the knowledge and capacity for ASF control in Ukraine. The project's Scientific Advancement activities integrated data to understand how ASF spreads, as well as how this relates to the nature of virulence, and to suggest means of control of ASF. Publication of project research and methodology by scientists in Ukraine, Poland, and the US will contribute to the scientific community's understanding of the virulence of ASFV/Georgia/2007 lineage strains that have spread and threaten to become endemic in Ukraine and other countries. Moreover, project-generated data on co-infections and epidemiological parameters can serve as a resource to inform and thus strengthen ASF control measures by state veterinary services and institutions in Ukraine.

2.2.4. Project Participants

- The State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE), Kyiv, Ukraine
  Principal Investigator: Andrii A. Mezhenskyi, Director

- National Scientific Center "Institute of the Experimental and Clinical Veterinary Medicine" (IECVM), Kharkiv, Ukraine
  Principal Investigator: Anton Gerilovych, Deputy Director

- Institute of Veterinary Medicine (IVM), Kyiv, Ukraine
  Principal Investigator: Oleksandr Tarasov, Head of Laboratory

- University of Alaska Anchorage (UAA), Anchorage, Alaska, USA
  SME: Eric Bortz, PhD, Assistant Professor, Dept. of Biological Sciences

- University of Alaska Fairbanks (UAF), Fairbank, Alaska, USA
  SME: Devin Drown, PhD, Assistant Professor

- Joint Genome Institute (JGI), Berkeley, CA, USA
  SME: Inna Dubchak, PhD

- National Veterinary Research Institute (NVRI), Puławy, Poland
  SME: Grzegorz Wozniakowski, PhD. ScD

- Metabiota Inc. (Metabiota), USA
  SME: Christian E. Lange, PhD/DVM

2.3. Technical Approach

2.3.1. Methodology

As a collaborative project among three scientific institutes in Ukraine (SSRILDVSE, the lead institution; IECVM; and IVM), UP-9 OY1 research included: (1) Laboratory diagnostics and ASFV genome sequencing, (2) epidemiological data analysis, and (3) science advancement activities.

The project's collaborators sequenced the full genomes of at least 12 ASFV strains and characterized an additional 64 strains by amplicon (partial) genome sequencing using MinION technology. Samples collected from domestic pigs and wild boar during outbreaks occurring in 2012-2020 were compared to ASFV isolates from other countries in the region (Poland, the Baltics, Georgia, Armenia, etc.). Differential signature sequences in the ASFV genome, which were identified during the project's base period, allowed for tracing the origin and spread of the virus. Additionally, genomics analysis of ASFV were combined with epidemiological analyses of factors governing infection to develop a more comprehensive picture of disease outbreaks and spread.

2.3.2. Description of Technical Approach

UP-9 base period activities provided a proof-of-principle that a novel nanopore sequencing (ONT MinION) approach could genotype ASFV from clinical samples (spleen) and sequence ASFV genome DNA. In OY1, this effort continued and expanded for broad ASFV genotyping and strain identification, epidemic tracing using genomic signatures, and understanding of variable genomic loci under host-selective evolutionary pressure as described below.

- A unique genomic signature for ASFV isolates was used for differential genotyping and evolutionary outbreak analysis of ASFV lineages mapped to season variation, outbreak region, host (wild boar or domestic pigs), and clinical and epidemiological data. Scientists at SSRILDVSE, in collaboration with UAA, were able to access the MinION nanopore sequencing platform for state-of-the-art next-generation sequencing analyses of pathogen genomes. A minimum of 64 archived and ongoing ASFV DNA samples from Ukraine were analyzed using this multiplex sequencing technology, providing a

genomic picture of the origins and spread of ASFV across a broad swath of Ukraine and transboundary regions.

- Select full genomes were sequenced on the MinION platform to observe genome-level evolution and analysis of pathogenesis, transmission, and temporal dynamics. All raw sequence data, genomic assembly (genotyping) data, and consensus genome sequences were released on NCBI GenBank after database management at SSRILDVSE and the University of Alaska.

- Development of expertise in virus next-generation sequencing techniques, bioinformatics data analysis and interpretation, and phylogenetic analysis were a highly relevant component of this pursuit. The project's SMEs coordinated the development of a research-focused, highly relevant curriculum using bioinformatics tools to support ASFV genome and evolutionary analysis. Training modules were developed both within the project and in coordination with the BTRP-Ukraine training program in order to ensure both the broad dissemination of materials and the development of competent Train-the-Trainer (T3) candidates.

These expanded ASFV genotype analyses provided information regarding the source and location of ASF introduction for individual outbreaks, the rate of spread within a country (with Ukraine the most studied example in this project), and, using phylogenetics analyses, the rate of evolutionary change. In addition, capacity building for nanopore sequencing, including bioinformatics education, allowed this analysis to be conducted in multiple participating countries in the region of the virulent ASFV/Georgia/2007 lineage outbreaks.

Epidemiological mapping of ASF cases in Ukraine, and comparison of ASF spread and control measures in Poland (in collaboration with researcher partners at NVRI, Puławy), allowed for a robust analysis of factors that may govern ASF outbreaks, spread, and relationships between genomic variation and pathogenicity in wild boar and domestic pigs. The dissemination of these findings at international meetings (ASF Stop/COST-Action in the European Union, Nanopore Community Meeting, American Society for Virology, etc.) and publication of scientific manuscripts aided in informing multinational efforts to control the spread of ASFV and, therefore, contributed significantly to the region's increased preparedness.

Activities for Scientific Advancement, including project coordination, bioinformatics capacity building, and mentorship, generated genomics expertise for nanopore sequencing of virulent ASFV. SSRILDVSE served as the project's lead Institute, working in collaboration with countries in the region, particularly Poland (NVRI). These activities permitted expansion of ASFV genomic analyses to regional partners.

2.4. Schedule and Milestones

2.4.1. Schedule

Yellow reflects the Quarter (Q) in which work initiated, green represents ongoing work (including work initiated during the base period), and dark blue represents completed work.

Milestones and Tasks	Q1: 01 Apr 2019 -30 Jun 2019	Q2: 01 Jul 2019 -30 Sept 2019	Q3: 01 Oct 2019 -31 Dec 2019	Q4+NCE*: 01 Jan 2020 -30 Jun 2020	Comments
1. Understand genotypes of ASFV in Ukraine and track the spread of virus by genotypic signatures.					
1.1. Trace origin and spread of ASF outbreaks in Ukraine (2014-2020) by genomic signature and full genome sequencing analyses of ASFV using nanopore sequencing (ONT MinION) technology.				Completed	Though two major outbreak origins were potentially identified, COVID-19 restrictions limited in-depth analysis of the current outbreak in western Ukraine and eastern Poland.
1.2. Understand ASFV virulence in wild boar and domestic pigs by genomics and evolutionary analysis of ASFV pathogenicity genes and detection of co-infection in swine.				Completed	



Milestones and Tasks	Q1: 01 Apr 2019 -30 Jun 2019	Q2: 01 Jul 2019 -30 Sept 2019	Q3: 01 Oct 2019 -31 Dec 2019	Q4+NCE*: 01 Jan 2020 -30 Jun 2020	Comments
2. Undertake investigations into the epidemiology of ASF in Ukraine to understand exposure, incidence, and prevalence for mapping the disease.					
2.1 Estimate the roles of environmental risk factors on incidence, persistence and geographic distribution of ASF outbreaks.				Completed	
2.2. Understand ASFV exposure and prevalence in ASF outbreak zones by serological surveillance of domestic pigs and wild boar.				Completed	
3. Scientific Advancement: Bioinformatics capacity-building and data sharing.					
3.1 Advance scientific capacity for pathogen genomic analysis through bioinformatics and sequence analysis workshops.				Completed	
3.2 Advance ASF data sharing, reporting, and international collaboration through workshops, communication among institutes in Ukraine and SMEs, and scientific presentations and publications. Please see subtasks below.				Completed	
3.2.1. Building regional partnerships for ASF genomics and control.					
3.2.1a. Potential regional partners identified during the UP-9 base period will be fully briefed on project				Completed	




Milestones and Tasks	Q1: 01 Apr 2019 -30 Jun 2019	Q2: 01 Jul 2019 -30 Sept 2019	Q3: 01 Oct 2019 -31 Dec 2019	Q4+NCE*: 01 Jan 2020 -30 Jun 2020	Comments
goals, activities, and opportunities, and subsequently, their general interest in participating will be confirmed.					
3.2.1b. The capabilities of regional partners for ASF investigation will be ascertained (e.g., does the regional partner currently perform PCR, genome sequencing, etc.).				Completed	
3.2.1c. The level of participation will be determined for each regional partner based on the partner's interest and capability (as informed by 3.2.1a and 3.2.1b)				Completed	
3.2.1d. Work flow for regional partners' participation will be established and communicated accordingly to each partner and the Ukrainian UP-9 OY1 team.				Completed	
3.2.1e. Potential new partners will be identified, briefed, and their level of participation will be determined				Completed	
3.2.2. *Scientific Advancement workshops and active interactions planned with regional partners.*					
3.2.2a. ASFV Advanced Genomics Workshop.				Completed	
3.2.2b. ASF Outbreak Tracing, Serology and Co-infection Workshop.				Completed	
3.2.2c. ASF Epidemiology Workshop.				Completed	

Note: NCE* - No-Cost Extension, 01 April – 30 June 2020

2.5. Project Presentations and Publications

2.5.1. Publications

- Kovalenko G, Ducluzeau AL, Ishchenko L, Sushko M, Sapachova M, Rudova N, Solodiankin O, Gerilovych A, Dagdag R, Redlinger M, Bezymennyi M, Frant M, Lange CE, Dubchak I, Mezhenskyi AA, Nychyk S, Bortz E, Drown DM. **Complete Genome Sequence of a Virulent African Swine Fever Virus from a Domestic Pig in Ukraine.** ASM Microbiol Resource Announc. 2019 Oct 17;8(42): e00883-19. doi: 10.1128/MRA.00883-19. PMID: 31624164; PMCID: PMC6797529 (see Appendix C, item 1).
- Mazur-Panasiuk N., Woźniakowski G., Niemczuk K. **The First Complete Genomic Sequences of African Swine Fever Virus Isolated in Poland.** Scientific Reports 9, 4556 (2019). *[This manuscript was not generated by UP-9 project activities per se; however, it was written in parallel and contributed significantly to ASFV genome annotation for MinION sequencing under UP-9 OY1, as well as to validating the short-read next-generation sequencing methodology described at NVRI].*
- UP-9 Derived Manuscripts in Preparation:
    - Sushko M., *et al*. Phyloepidemic tracing of African swine fever virus (ASFV) in Ukraine from long read nanopore sequencing. *(manuscript in preparation; for* PLoS One*).*
    - Bezyemenni M., *et al*. Spatio-temporal outbreak model of the spread of African swine fever in Ukraine, 2014-2020. *(manuscript in preparation; for* Vector-Borne & Zoonotic Diseases*).*
    - Rudova N., Buttler J., O. Solodiankin, *et al.* Genetics of porcine circovirus-2 distribution in Ukraine. *(manuscript in preparation; for* Vector-Borne & Zoonotic Diseases*).*
    - Arafaev V., SolodiankinO., *et al.* Isolation and genome analysis *Salmonella suis* from a swine-product market in Ukraine. *(manuscript in preparation; for* ASM MRA*).*

2.5.2. Meetings and Presentations

- Two poster presentations at the ASM Biothreats meeting, 29-31 January 2019, Arlington, USA (see Appendix C, items 2-3)
    - Poster (presented by Bezymennyi M., IVM): **Spatial Analysis of the Spread of African swine fever in Ukraine**;
    - Poster (presented by Sapachova M., SSRILDVSE): **Genomic sequencing of virulent African swine fever virus in Ukraine**
- Oral presentation at the American Society for Virology's 38th Annual Meeting (Bortz E. *et al*.), 20-24 July 2019, Minneapolis, USA (see Appendix C, item 4): **Conserved Genome Architecture of Virulent African Swine Fever Virus (ASFV) in Ukraine from Long Read Nanopore Sequencing**

- Oral and poster presentations at the DTRA Science Program Review, 19-20 September 2019, Warsaw, Poland:
  - Poster (presented by Mezhenskyi A., SSRILDVSE): **Integration Across BTRP-Funded ASF Mitigation Activities to Reduce the Threat of Transboundary Disease**
  - Poster (presented by Gerilovych A., IECVM): **Strengthening Ukraine's Veterinary Biosurveillance System through Collaborations Developed within the CBR Project UP-9**
  - Poster (presented by Kovalenko A., IVM): **Next Generation Sequencing within UP-9: Highlighting the Relevance of Genomics Data for Biological Threat Reduction and Biosurveillance Systems**
  - Oral Presentation (presented by Kovalenko A., IVM; see Appendix C, item 5): **The Spread of African Swine Fever Virus (ASFV) in Domestic Pigs and Wild Boar in Ukraine – Building Capacity for Insight into the Transmission of ASFV through Characterization of Virus Isolates by Genome Sequencing and Phylogenetic Analysis**
- Poster presentations at the Fourth Annual BTRP Ukraine Regional One Health Research Symposium (ROHRS), 20-24 May 2019, Kyiv, Ukraine (see Appendix C, items 6-8):
  - Poster 1 (presented by Sapachova M., SSRILDVSE): **Bioinformatics Efforts as a Part of UP-9 Project Implementation within Cooperative Biological Engagement Program in Ukraine**
  - Oral Presentation/Poster (presented by Sushko M., SSRILDVSE): **Nanopore (MinION) Sequencing of African Swine Fever Virus in Ukraine**
  - Oral Presentation/Poster (Gerilovych A., Solodiankin S., Rudova N.; IECVM). **UP-9 OY1 Project Methods and Results for Discussion with Ukrainian Peer Scientists**
- Abstracts submitted for 2020 ROHRS (this conference is suspended due to the COVID-19 pandemic):
  - **Nanopore (MinION) Sequencing of Porcine Circovirus type 2 in Ukraine** (see Appendix C, item 9)
- **Scientific Advancement Workshops.** Ukrainian and regional participating scientists presented ASF and co-infection research progress regarding UP-9 and UP-9 OY1 activities via advanced learning and regional collaboration workshops held in Ukraine and Poland. Workshops focused on epidemiological analysis, advanced bioinformatics skills, MinION nanopore sequencing, and epidemiological data analysis. Through these workshops, partnerships were strengthened for regional ASF control, research activities and communication on epidemiological data sharing and virus genomics, and risk analysis methods. Follow-on activities were conducted electronically. Participants from four institutes in Ukraine (SSRILDVSE, IECVM, IVM, and UAPRI), one in Poland (NVRI), three institutes in Georgia (Georgia CDC, Ilia State University, Lab MoA), and MoA- and university-affiliated labs in

Armenia and Moldova, together with SMEs from the University of Alaska, built a nascent threat reduction network for communication and control of ASF. Workshops included:

- **Bioinformatics and Epidemiology Data Workshop** (27-31 May 2019; IVM and SSRILDVSE, Kyiv, Ukraine).
- **Genomics and Concurrent Infection Workshop** (18-21 June 2019; IECVM, Kharkiv, Ukraine).
- **UP-4/UP-9 Transboundary Virus Epidemiology and Sequencing Workshop** (09-13 September 2019; SSRILDVSE and IVM, Kyiv, Ukraine).
- **Workshop and Regional Collaboration in Outbreak Tracing using Genomics and GIS-Based Epidemiological Analyses for African Swine Fever (UP-9)** (9-13 December 2019; SSRILDVSE and IVM, Kyiv, Ukraine).
- **Ukraine-Poland Joint Workshop** with Dr.Grzegorz Wozniakowski's team (16-17 September; NVRI, Puławy, Poland).
- **UP-9/UP-9 Synergy Workshop** was scheduled for April 2020 in Kyiv, Ukraine, but was cancelled due to COVID-19 pandemic-related restrictions. A very limited set of activities was undertaken electronically.

- **Training events conducted in coordination with the BVSPC Training Team.**
  - **Theoretical and practical bioinformatics workshop** was conducted by Jana Schulz from Friedrich-Loeffler-Institut (FLI; 03-07 June 2019; SSRILDVSE, Kyiv, Ukraine).
  - **Training course on African swine fever diagnostics, genetic characterization and bioinformatics** (24 June-04 July 2019; FLI, Greifswald, Germany).
  - **One Health Workshop** № 13 – An Overview of Bioinformatics Tools, Mentorship Session (09-13 December 2019; Kyiv, Ukraine).
- **Virtual Project Close-Out Workshops.** Ukrainian and regional participating scientists met to review and discuss findings and accomplishments regarding ASF surveillance, including efforts focused on, but not limited to, capacity development, surveillance actions, forecasting, amendments to regulations for regional data sharing in support of an ASF collaborative network, and strategies for continued regional collaborative activities.
  - **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (15 June 2020), Microsoft Teams Platform, Kyiv, Ukraine
  - **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (16 June 2020), Microsoft Teams Platform Kyiv, Ukraine

- **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (19 June 2020), Microsoft Teams Platform Kyiv, Ukraine
- **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (22 June 2020), Microsoft Teams Platform Kyiv, Ukraine
- **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (25 June 2020), Microsoft Teams Platform Kyiv, Ukraine
- **UP-9 OY1/UP-10 CBR Project Close-Out Meetings Virtual Conferences** (30 June 2020), Microsoft Teams Platform Kyiv, Ukraine

2.6. Technical Report

2.6.1. Findings Against Planned Objectives

Aim 1. Understand genotypes of ASFV in Ukraine and track the spread of virus by genotypic signatures.

- ***Task 1.1:*** Trace origin and spread of ASF outbreaks in Ukraine (2014-2020) by genomic signature and full genome sequencing analyses of ASFV using nanopore sequencing (ONT MinION) technology
- ***Task 1.2:*** Understand ASFV virulence in wild boar and domestic pigs by genomics and evolutionary analysis of ASFV pathogenicity genes and detection of co-infection in swine

Results and Discussion on Task 1.1

Results:

ASFV full genome sequencing and epidemic tracing: The UP-9 team undertook research on genomics, epidemiology, and risk mapping of ASFV and related co-infections. Major laboratory research activities were focused on:

- Sequencing and bioinformatics analysis of ASFV full genome sequences.
- Sequencing of swine co-infection pathogens using PCR-based tiling primer amplification and full DNA viromics approaches.
- Developing and training in advance bioinformatics methods for raw genomic data analysis and genome assembly of ASFV and co-infection pathogens sequences.
- Phylogenetics of virus genomes using comparative evolution methods for tracing the origin and spread of the virulent ASFV/Georgia/2007 p72 genotype II lineage in Ukraine and the region.

ASFV full genome sequencing: 14 strains sequenced. Thirteen ASFV isolates were sequenced by the collaborative UP-9 project team using the MinION device in Ukraine, as well as one strain in Poland, with one genome and associated methods published (**Table 1**). Strains for sequencing were selected to cover early outbreaks (2014) to current outbreaks (2019), and to focus on "hotspots" for ASF emergence, as posited by the UP-9 OY1 epidemiological analysis team. This included an analysis of wild boar and domestic pig ASFV isolates from the central and western regions of Ukraine that are sources of continual re-emergence of ASFV. The origin of the virulent strain in a large transboundary outbreak in western Ukraine (Lviv) and eastern Poland in 2019 was unknown. One contemporary strain from a large commercial farm outbreak in western Ukraine near Lviv (2019) was sequenced (#12, outbreak #498), and one from across the border in Poland (#14), to understand potential transboundary links between these outbreaks and threat to commercial swine production (**Table 1**).

Accurate ASFV genome sequencing by MinION in Ukraine. The UP-9 team undertook bioinformatics analysis of ASFV full genome sequences, including analysis of systematic errors in Illumina short-read and nanopore long-read sequencing of genomes to improve accuracy of reported ASFV genotypes and variants. Highly accurate genome sequencing of ASFV was achieved through technical improvement of methods. Genome coverage ranges from 24X-199X (reads per nucleotide of virus DNA genome sequence). With improved basecalling (ONT *Guppy*) software, and improved R9.4 flow cells (hardware), a read depth of 28-30X is sufficient to eliminate random errors in nanopore (MinION) sequence raw reads [see UP-9 publication, ref. #8]. However, bioinformatics analysis of systematic (homopolymer) errors requires inspection (manual correction). New versions of ONT *Guppy* software and genome assembly methods applying quality control (QC) software (*Flye, Racon, and Medaka*) have provided highly accurate genome sequencing of ASFV genomes using MinION devices in Ukraine. In addition, the UP-9 team tested new MinION R10 flow cell technology, viral DNA capture library preparation (REPLI-G methods), and improvements to the library preparation protocols. These were tested in Alaska on a large DNA virus (EBV), a technical model for ASFV, reducing raw data error rates considerably, and taught and applied during workshops in Ukraine for regional sequencing efforts.

Detailed methods for MinION sequencing of ASFV. Rapid DNA library preparation and application of MinION for full viral genome nanopore sequencing on a test clinical isolate, an ASFV strain from a domestic pig (outbreak #131, Kyiv Oblast, November 2016), was conducted by Ukrainian scientists at IVM, with collaborators from SSRILDVSE and IECVM, as well as guidance from SMEs (University of Alaska). After genome assembly (**Fig. 1**), analysis was conducted for the whole-genome sequence to identify signature sites of nucleotide variation (SNP/INDEL) for epidemic tracing of the pathogen.

These data resulted in the first publication of an ASFV genome in Ukraine and the first publication of a method for ASFV genome sequencing from a clinical isolate using MinION methods [8]. This isolate, ASFV/Kyiv/2016/131, is a virulent strain of the ASFV/Georgia/2007 p72-genotype II lineage (**Table 2**). The bioinformatics analysis method developed in this pilot experiment was used as a template for the genome correction method for all ASFV whole-genome sequences generated throughout the project, resulting in 14 genomes (**Table 2**).

Table 1. List of isolates of ASFV sequenced by MinION.

№	ASF Outbreak Strain	Reads Mapped	Bases Mapped	Coverage
1	ASFV/Chernigov/2014/7	1,107	5 Mbases	24x
2	ASFV/Zakarpattya/2017/243	2,437	16 Mbases	77x
3	ASFV/Odesa/2018/416	1,105	5 Mbases	28x
4	ASFV/Poltava/2018/375	1,495	6 Mbases	34x
5	ASFV/Mykolaiv/2015/53	1,582	7 Mbases	40x
6	ASFV/Kyiv/2016/131*	2,849	7 Mbases	32x
7	ASFV/Chernivtsi/2018/351	33,376	39 Mbases	199x
8	ASFV/Kyiv/2018/434	1,685	6 Mbases	33x
9	ASFV/Cherkasy/2018/369	717	4 Mbases	24x
10	ASFV/Chernivtsi/2018/328	4,981	23 Mbases	119x
11	ASFV/Kyiv/2018/422	5,348	18 Mbases	94x
12	ASFV/Lviv/2019/498	Analysis in progress		
13	ASFV/Mykolaiv/2019/501	Analysis in progress		
14	ASFV/Poland/2019 [NVRI]	Analysis in progress**		

Notes:

* Sequencing method using MinION, bioinformatics analysis, and strain annotation for ASFV/Kyiv/2016/131* (GenBank Accession: MN194591.1) was published in Q2 [ref. #8].

** MinION sequencing method is in development at the NVRI lab (Puławy, Poland). Previous experience using Illumina sequencing and ASFV genome annotation [ref. #9] contributed to laboratory-based investigation of novel strains using MinION for hybrid genome assembly.

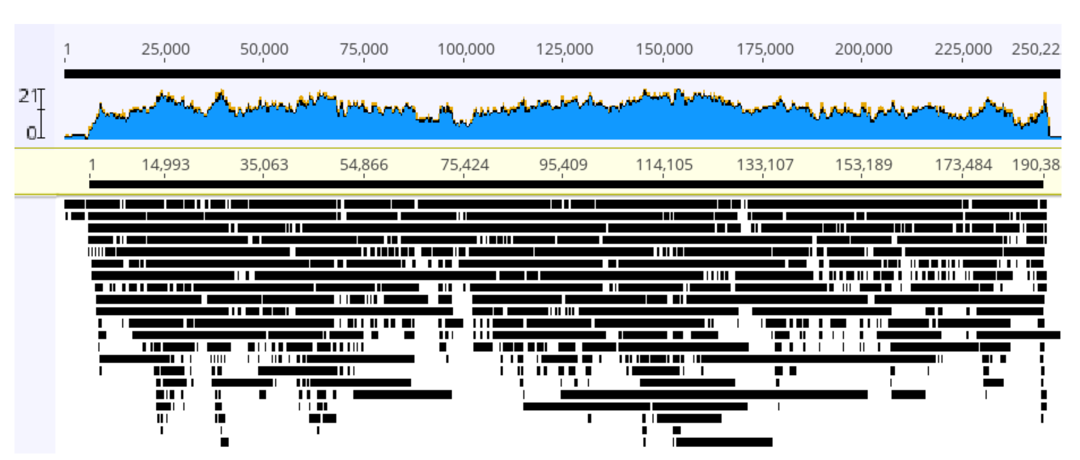


Figure 1: Complete genome sequencing of ASFV using MinION. ASFV DNA sample was prepared using the ONT Rapid total DNA sequencing library method and analyzed on an R9.4 flow cell on a MinION device at SSRILDVSE (Kyiv, Ukraine; Sushko M. Bezyemenni M., Drown D.). The draft assembly of the ASFV genome (189kbp) from ASF outbreak #501 (Mykolaiv, 2019) from 25% of the raw data indicates full genome coverage (black bars, individual long sequence reads).

Table 2. Nucleotide identity (% bases/residues, which are identical) between different whole-genome ASFV isolates from Ukraine, Georgia, China, and Belgium.

ASFV isolates	ASFV/Kyiv/2016/131	Georgia 2007/1 FR682468	ASFV/POL/2015/Podlaskie MH681419	China/2018/AnhuiXCGQ MK128995	Belgium 2018/1 LR536725
ASFV/Kyiv/2016/131	-	99.950%	99.964%	99.980%	99.595%
Georgia 2007/1 FR682468	99.950%	-	99.946%	99.948%	99.950%
ASFV/POL/2015/Podlaskie MH681419	99.964%	99.946%	-	99.971%	99.971%
China/2018/AnhuiXCGQ MK128995	99.980%	99.948%	99.971%	-	99.986%
Belgium 2018/1 LR536725	99.595%	99.950%	99.971%	99.986%	-

UP-9 OY1 scientists in Ukraine led sequencing efforts. Critically, with guidance from UAA and UAF SMEs, scientists in Ukraine, Poland, and regional collaborators have led sequencing efforts using MinION-based genomics for ASFV and other (co-infection) pathogens (**Fig. 2**). ASFV was successfully sequenced using MinION methods at two institutes in Ukraine (SSRILDVSE and IVM; Kyiv, Ukraine) and one in Poland (NVRI; Puławy, Poland). Swine co-infection pathogens were successfully sequenced using MinION at SSRILDVSE and IVM (Kyiv, Ukraine), IECVM (Kharkiv, Ukraine), and the University of Alaska (for control strains). Ukrainian scientists independently sequenced ASFV and co-infecting swine pathogens during workshops, with only occasional guidance as




needed by project SMEs. As MinION sequencing and advanced bioinformatics skills progressed throughout UP-9 OY1, lead Ukrainian and Georgian bench researchers adopted a Train-the-Trainer method of guiding fellow scientists for Ukraine and regional partners. With frequent continuing communication with each other and project SMEs at the University of Alaska, these up-and-coming scientists have formed the core of a nascent regional threat reduction network for pathogen genome sequencing using MinION and advanced bioinformatics analysis, applicable to ASFV, swine co-infections, and other wildlife, livestock and human pathogens.




Figure 2: UP-9 OY1 laboratory teams after successfully completing MinION sequencing runs. Ukrainian, Georgian, and Moldovan researchers, as well as SMEs, after ASFV and swine co-infection pathogen sequencing on MinION devices at IVM (*left*) and SSRILDVSE (*right*) during workshops in Kyiv, Ukraine (2019).

Sequence data analysis, archiving, and reporting. All sequence data are stored at the IVM server and backed up at the University of Alaska. Computational quality control (QC), assembly, and annotation of complete genomes is in progress, with Ukrainian and regional network researchers learning and applying bioinformatics methods for virus genome analysis. ASFV genomes will be reported in one or more publications and additional submissions to GenBank. One ASFV genome is already deposited in GenBank (**Table 1;** GenBank Accession: MN194591.1), and others will follow a similar format (under NCBI BioProject PRJNA555080).

Discussion:

Phylogenetics-based epidemic tracing of ASFV outbreaks in Ukraine. The UP-9 team has sequenced isolates of virus from the main epidemic clusters identified by previous epidemiological analyses, including border and interior regions. Sequences were generated from the first (2014) and the latest (2019) outbreaks of ASF in Ukraine (**Table 1**). A key outcome of sequencing ASFV genomes from across the epidemic in Ukraine has been the development of a genomic tracing method to map and trace links between outbreaks by combining GIS-based ASF

outbreak data and ASFV genome sequence analysis by phylogenetics (**Fig. 3**). According to phyloepidemic tracing analysis, closely-related ASF outbreaks clustered temporally, one series of outbreaks with weak geographic clustering in central and southern Ukraine in 2017-2018, and an earlier independent introduction of ASF in central and northern Ukraine in 2014-2018 (**Fig. 3**). Therefore, this analysis potentially identified two major outbreak origins, or transboundary events, that introduced ASF into Ukraine, resulting in subsequent spread across the country. Additional introductions likely also occurred, as some ASFV genomes do not cluster with either of the two major groups. A manuscript describing epidemic spread of ASF in Ukraine by analysis of genomic signature in these ASFV genomes (and other partial genomes by PCR/amplicon sequencing), is in preparation (Sushko M., *et al*. *Phyloepidemic tracing of African swine fever virus [ASFV] in Ukraine from long read nanopore sequencing*). Analysis of the large ongoing ASF outbreak in western Ukraine and eastern Poland was initiated but not completed due to the interruption in research activities caused by the COVID-19 pandemic beginning March 2020.

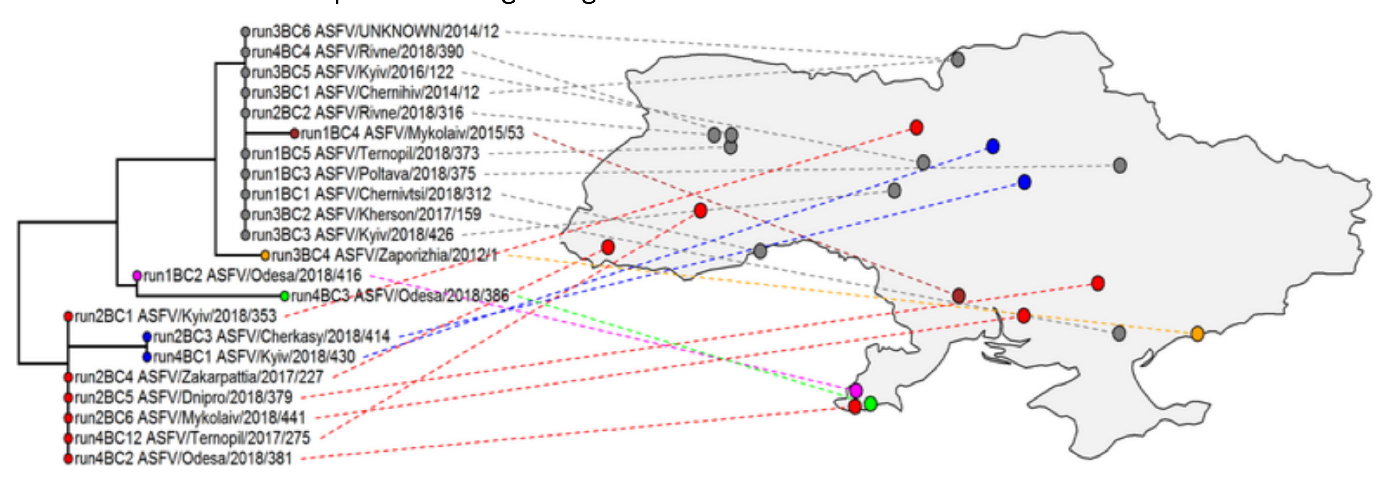


Figure 3: Phyloepidemic tracing of ASF outbreaks in Ukraine. ASFV DNA was sequenced using MinION devices in Ukraine and analyzed by a neighbor-joining (NJ) phylogenetic tree. Closely-related sequences on the tree cluster temporally with weak geographic clustering in central and southern Ukraine (red dots 2017-2018), or central and northern Ukraine (grey dots 2014-2018), potentially representing two major origins of outbreaks or transboundary events introducing ASF into Ukraine. Additional introductions also occurred (other colored dots).

Results and Discussion on Task 1.2

Analysis of ASFV pathogenicity genes for tracking ASF spread. A deep bioinformatics analysis of sequenced genomes makes it possible to identify changes in the viral genome characteristic for a particular cluster, thereby allowing for tracking the origin of the virus. Identification of character changes in

pathogenicity genes in the ASFV genome, in combination with epizootological data, provide an opportunity to better understand the mechanisms of spread and vectors of virus transfer.

Bioinformatics and phylogenetics approach. A phylogenetics-based approach using comparative evolution methods of ASFV multi-gene family (MGF) was developed and used for understanding the origin of the virulent ASFV/Georgia/2007 p72 genotype II viruses, the lineage of ASFV that has spread in Ukraine and the region. The multigene families include groups of genes from ASFV and contain at least five members: MGF100; MGF110; MGF300; MGF360; MGF505; MGF genes associated wwith swine virulence and host range and have been identified in the terminal variable regions of the ASFV genome. The MGF member of each ASFV isolate includes a specific number of these genes. A multigene family is a member of related proteins encoded by a set of similar genes, supposedly resulting from duplication and variation of a single ancestral gene. DNA duplications can generate gene pairs, and as a result, both copies are saved in subsequent virus generations as a multigene family, dispersing multigene families throughout the genome.

Researchers at IVM, SSRILDVSE, and IECVM in Ukraine, NVRI in Poland, and the University of Alaska collaborated in this analysis. MGF protein sequences (coding sequences) of all five MGF members from 23 ASFV complete genome isolates were extracted (NCBI GenBank collection with annotations, **Table 3**). Due to inconsistencies in genome databases and ASFV scientific literature, MGF were manually identified under alternate nomenclatures from two relevant families, MGF 360 (**Table 4**) and MGF505 (**Table 5**). The MGFs from each member of each ASFV isolate were translated and concatenated [110MGF, 100MGF, 360MGF, 300MGF, 505MGF], then aligned with the MAFFT algorithm in *Geneious* R11 software. Phylogenetic analyses of the 114 concatenated protein sequences were performed using Geneious Tree Builder with Neighbor-Joining (NJ) method and Jukes-Cantor genetic distance model (1000 bootstraps). In addition, each MGF member was analyzed separately using the IQ-TREE phylogenomic web-server by maximum likelihood (ML) with the ultrafast bootstrap (1000) branch supports. Appropriate substitution models were determined through the software options, and the divergence among amino acid sequences were modeled. The trees were summarized, visualized, and annotated in *FigTree* v1.4.4. Cladograms with increasing node order were constructed and colored to depict each clade or subgroup of MGFs (**Fig. 4**, additional draft trees not shown).

Table 3. Protein sequences of MGF members from ASFV isolates used in phylogenetics.

#	ASFV isolate name	#GenBank	Genotype	Host	Year	Country	Number of MGF's members (CDS)					Ref
							110	100	360	300	505	
1	Georgia_2007/1	FR682468	II	dp	2007	Georgia	12	2	16	2	10	Chapman,D.A., et al, 2011
2	Georgia 2008/1	MH910495	II	n/a	2008	Georgia	11	2	14	2	10	Farlow,J., et al, 2018
3	Estonia_2014	LS478113	II	wb	2014	Estonia	1	2	17	3	10	Submitted by Friedrich-Loeffler-Institute, 2018
4	Pol17_04461_C210	MG939588	II	wb	2017	Poland	8	2	14	-	9	Wozniakowski,G. (Unpublished)
5	Pol16_20186_o7	MG939583	II	wb	2016	Poland	10	2	16	2	9	Wozniakowski,G. (Unpublished)
6	ASFV/LT14/1490	MK628478	II	wb	2014	Lithuania	12	3	19	3	10	Gallardo, C., et al, 2017
7	**ASFV/Kyiv/2016/131**	**MN194591**	II	**dp**	**2016**	**Ukraine**	**11**	**3**	**19**	**4**	**13**	Kovalenko,G., et al, 2019
8	Belgium/Etalle/wb/2018	MK543947	II	wb	2018	Belgium	10	3	16	3	11	Gilliaux,G., et al, 2019
9	Belgium 2018/1	LR536725	II	n/a	2018	Belgium	14	3	19	3	10	Submitted by Friedrich-Loeffler-Institute, 2019
10	ASFV-wbBS01	MK645909	II	wb	2018	China	11	2	16	1	10	Zhaowen,R. (Unpublished)
11	China/2018/AnhuiXCGQ	MK128995	II	dp	2018	China	12	2	16	2	10	Bao,J., et al, 2019
12	ASFV/pig/China/CAS19-01/2019	MN172368	II	dp	2019	China	11	2	15	2	10	Jia,L., et al, 2019
13	CzechRepublic 2017/1	LR722600	II	n/a	2017	Czech Republic	12	3	19	3	10	Submitted by Friedrich-Loeffler-Institute, 2019
14	Moldova 2017/1	LR722599	II	n/a	2017	Moldova	12	3	19	3	9	Submitted by Friedrich-Loeffler-Institute, 2019
15	E75	FN557520	I	dp	1975	Spain	5	2	16	4	10	de Villiers,E.P., et al, 2010
16	Benin_97/1	AM712239	I	dp	1997	Benin (Africa)	5	2	16	4	10	Chapman,D.A., et al, 2008
17	L60 (high-virulence)	KM262844	I	dp	1960	Portugal	6	2	16	3	9	Portugal,R., et al,2015
18	OURT_88/3 (avirulent field isolate)	AM712240	I	tick	1988	Portugal	7	3	11	3	8	Chapman,D.A., et al, 2008
19	47/Ss/2008	KX354450	I	dp	2008	Italy	6	2	17	4	10	Granberg,F., et al, 2016
20	NHV (low-virulence)	KM262845	I	dp	1968	Portugal	7	3	11	3	8	Portugal,R., et al, 2015
21	Ken06.Bus	KM111295	IX	dp	2006	Kenya	12	3	15	3	9	Bishop,R.P., et al, 2015
22	R35	MH025920	IX	dp	2015	Uganda	14	4	17	3	9	Masembe,C., et al (Unpublished)
23	Ken05/Tk1	KM111294	X	tick	2005	Kenya	13	3	18	3	9	Bishop,R.P., et al, 2015

Table 4. List of MGF 360 alternative names.

Name	Other Name	Name	Other Name	Name	Other Name
MGF 360-1L	KP360L	MGF 360-9L	A125L, 3CL	MGF 360-17R	Not found
MGF 360-2L	KP362L	MGF 360-10L	Not found, 3DL	MGF 360-18R	DP148R
MGF 360-3L	L356L	MGF 360-11L	Not found, 3EL	MGF 360-19R	DP363R
MGF 360-4L	Not found	MGF 360-12L	Not found, 3HL	MGF 360-20R	DP42R
MGF 360-5L	Not found	MGF 360-13L	Not found, 3IL	MGF 360-21R	Not found
MGF 360-6L	Not found	MGF 360-14L	Not found, 3LL	MGF 360-22R	Not found
MGF 360-7L	Not found	MGF 360-15R	A276R		
MGF 360-8L	J319L	MGF 360-16R	DP311R		

Note: Names were identified via The UniProt Consortium 2018, under the ASFV strain Ba71V (Badajoz 1971, Vero-adapted), except for 3CL, 3DL, 3EL, 3HL, 3IL, 3LL, which can be found in Burrage *et al*., 2004.

Table 5. List of MGF 505 alternative names.

Name	Other Name	Name	Other Name
MGF 505-1R	Not found, L3FR	MGF 505-7R	A528R
MGF 505-2R	A489R, L3NR	MGF 505-8R	Not found
MGF 505-3R	A280R, L3QR	MGF 505-9R	A506R
MGF 505-4R	A505R	MGF 505-10R	A524R
MGF 505-5R	A498R	MGF 505-11R	DP542L, L230L
MGF 505-6R	Not found		

Note: Names were identified from The Uniport Consortium (2018) and from Yozawa *et al.,* 1994.

Implications. This analysis suggests that the virulent ASFV/Georgia/2007-lineage (p72 genotype II) of viruses currently affecting Eurasia is evolutionarily distinct from ASFV strains in Africa and previously identified in Europe, forming a separate MGF clade (**Fig. 4**). Moreover, the method for phylogenetics analysis of ASFV genomes is being used for epidemic tracing of ASF outbreaks in Ukraine and the region by identification of outliers (variations) in MGFs. These data will be reported in a manuscript in preparation (Sushko M., *et al*. *Phyloepidemic tracing of African swine fever virus [ASFV] in Ukraine from long read nanopore sequencing*). Expansion of these efforts could potentially be considered, but via a separate study, taking into consideration work interruptions stemming from the COVID-19 pandemic, which impacted further UP-9 analyses.

Figure 4. ASFV MGF phylogenetics. Concatenated protein sequences of all five MGF members from 23 ASFV complete genome isolates, illustrating the deep evolutionary history of ASFVs. The cladogram shows increased node order and colors regarding the MGF member name, highlighting evolutionary clades. These data will contribute to understanding the basis of transmission of virulent ASFV in Ukraine and Eurasia.

Analysis of swine co-infections. To better understand the potential effects of other swine pathogens on the susceptibility of domestic swine to ASF in Ukraine, a study of swine co-infections was undertaken. Samples provided primarily by IECVM, and along with IVM, SSRILDVSE, and UAPRI, were studied by diagnostic PCR and pathogen genome sequencing using MinION to understand swine co-infections that are circulating in Ukraine. Using specific PCR and laboratory-based virological and bacteriological analyses, swine co-infections were identified from biobanked domestic swine samples (swabs) archived at IECVM from symptomatic pigs. A limited number of samples were also obtained from pork production (local markets) to track co-infections in food production and risks of exposure of the human population in Ukraine. Methods for genome sequencing of diverse swine co-infecting viruses and bacteria were developed with the help of SMEs and then applied to understand such co-infections. Interestingly, both virus and bacterial co-infections were identified in pork products on sale in markets.

Pathogens that were analyzed included the following co-infecting viruses prevalent in Ukraine and the region: Porcine circovirus 2 (PCV2), porcine epidemic diarrhea virus (PEDV), porcine teschovirus (PTV1); as well as technical models for genomic differentiation of classical swine fever (a flavivirus), porcine paramyxoviruses, and bacterial infections (*Salmonella suis* spp. and model bacteria), which are clinically different for non-ASF hemorrhagic fevers in swine herds (personal communication, Dr. Anton Gerilovych, IECVM). Co-infection sequencing significantly contributed to training in pathogen genome sequencing and bioinformatics analyses for UP-9 researchers from Ukraine and the project's regional partners (**Fig. 5**). These efforts also contributed to improving data archiving, integrity, and transfer for facilitating bioinformatics analysis.



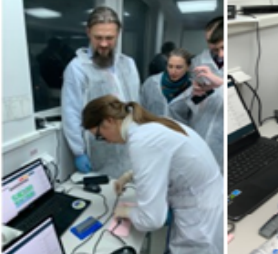
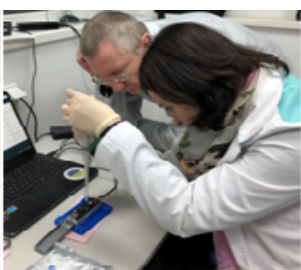




Figure 5. Application of laboratory methods for high accuracy swine co-infection pathogen sequencing. Study employed the MinION device and was led by project scientists at SSRILDVSE and IVM (Kyiv, Ukraine), as well as IECVM (Kharkiv, Ukraine).

Viral swine co-infection: *porcine circovirus 2* (PCV2). PCV2 can cause a lethal disease in swine, and although not infectious to humans, the virus can potentially contaminate pork production and spread to new locations through swine trade. A total of 18 samples identified as diagnostic PCR-positive were

sequenced; including 12 swine organ (spleen) DNA samples archived from a previous outbreak and 6 pork organ (liver) DNA samples purchased from a pork products market in Ukraine. Swine co-infection DNA was amplified specifically by PCR with genome-length tiling primers, and amplicons were nanopore multiplex-sequenced using MinION (**Fig. 6**). A primer map illustrates genome primer binding sites for PCV2 (developed by IECVM scientists in collaboration with SMEs at the University of Alaska [**Fig.7**]).

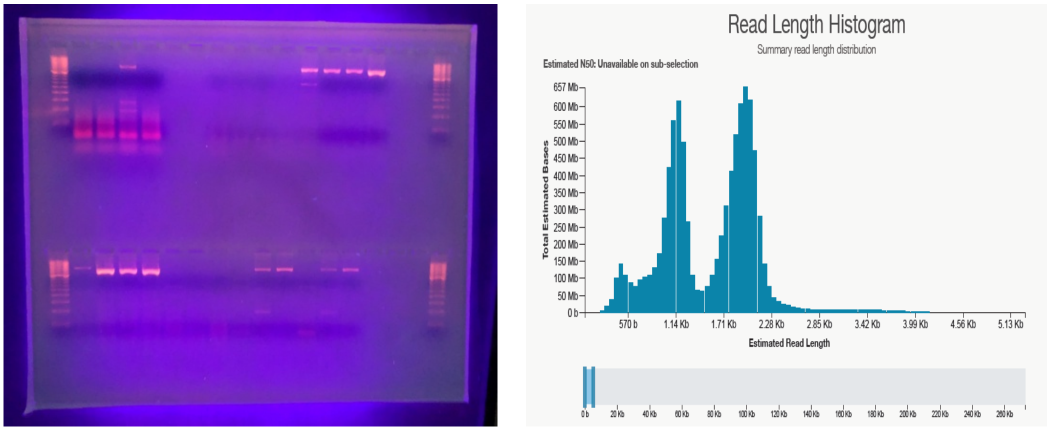


Figure 6. PCR amplification and MinION sequencing of amplicons from co-infections. Gel electrophoresis of amplicon library for PCV2, PEDV, and PTV1 (*left*). Histogram of MinION read length distributions showing clear size distributions of virus genome PCR amplicons at high read depth (*right*).



Figure 7. Genome diagram of PCV2 showing capsid binding and expected primer binding sites. Amplification for capsid and full genome sequencing of PCV2 are also depicted.

Phylogenetics analysis of PCV2. Nanopore sequences of PCV2 DNA isolated from archived samples were assembled and analyzed to understand virus genetics and phylogeographic origins. PCV2 capsid and full genome sequences were assembled to a PCV2 reference strain from Austria, 2003 (AY424401), to generate consensus sequences for phylogenetics analysis. Eleven (11) of the 12 sequences had a high quality read depth >100 reads per nucleotide. Consensus sequences for all 12 sequences covered the capsid gene of the PCV2 (nucleotides 960-1780 in the PCV2 genome). Multiple sequence alignment (MSA) and a phylogenetic tree were constructed using MAFFT (bootstraps: 1000) for the 11 highest quality capsid sequences (**Fig. 8**).

EF458306.1 Argentina
KJ920205.1 Germany 2011
KP231150.1 Italy 2010 dp
KY806044.1 England 2013 dp
KR559715.1 Italy 2011 wb
KP231112.1 Italy 2008 dp
KP231113.1 Italy 2009 dp
EF524534.1 China
KY656103.1 China
MG182445.1 China 2017
KP420193.1 Ukraine 2011 wb
KP420198.1 Ukraine 2010 wb
KT868521.1 USA 2006 wb
JN006463.1 Romania 2010 wb
JN382168.1 Romania 2011 wb
KY806069.1 England 2015 dp
KC620511.1 South Korea 2010 wb
KJ946351.1 Hungary dp
FJ644556.1 China 2005 dp
Poltava 1
Volyn 1
Zaporizhzhia 1
Chernivtsi 1
Chernivtsi 2
Chernihiv 1
Chernihiv 3
Chernihiv 2
Chernihiv 4
Luhansk 2
FJ233910.1 Canada 2007 dp
KP231139.1 Italy 2008 dp
JN006465.1 Romania 2010 wb
KP420202.1 Ukraine 2012 wb
KP420199.1 Ukraine 2012 wb
MH055412.1 China 2016 dp
MF139068.1 China 1997 dp
MF139063.1 China 1999 dp
KP420186.1 Ukraine 2011 wb
MF139057.1 China 1998 dp
Poltava 2
MF139078.1 China 1999 dp
Porcine circovirus 1 strain PK, complete genome.
genotipe b
Ukraine 2012
genotype a
genotype a
genotype f
0.05

Figure 8. Molecular epidemiology of PCV2 isolates in Ukraine. Eleven partial genome sequences of PCV2 (capsid gene) were analyzed using bioinformatics tools. Ten isolates from Zaporizhzhia, Chernovetsy, Poltava, Volyn, Lugansk, and Chernihiv Oblasts belonged to the B genotype, which is typical for Europe. One PCV2 isolate from Poltava Oblast was phylogenetically associated with a Chinese isolate of PCV2 (genotype F).

Most (11/12) of the Ukraine PCV2 were subtype B and belonged to a large clade that included PCV2 isolates from Austria, Slovakia, Hungary, Romania, and China. One strain was provisionally identified in the novel subtype F from China. These results suggest that while most PCV2 subtypes in domestic swine in Ukraine are transmitted in a regional context (a Central/East European group), long distance transfer of virus across Eurasia or from China may be possible (**Fig. 9**). Analysis of PCV2 in swine herds and pork products may indicate trade routes and risk for transmission of swine pathogens. In addition, 6 full genomes from pork products

were sequenced and are under analysis. These results highlight modes for spread of swine diseases, including PCV2, but potentially also ASF, swine influenza, and other co-infecting/concurrently infecting swine pathogens. A manuscript is in development for reporting these data (Rudova N., Buttler J., Solodiankin O., *et al*. *Genetics of porcine circovirus-2 distribution in Ukraine*. [*in preparation*]).

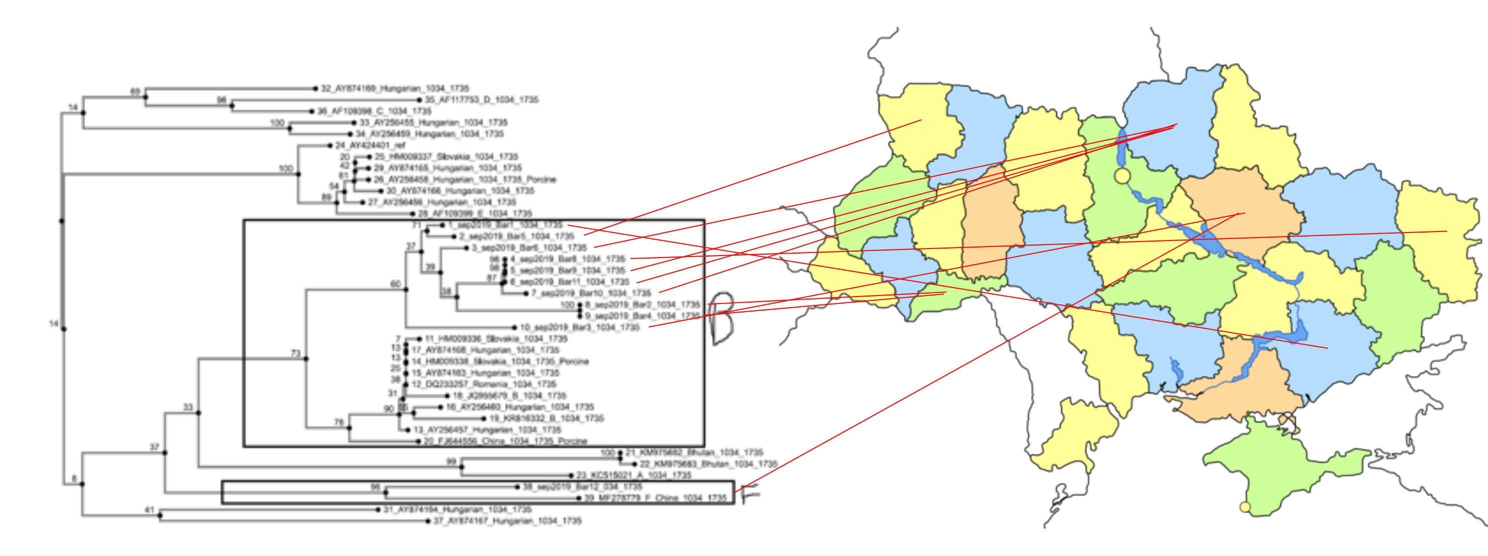


Figure 9. Phyloepidemic tracing of PCV2 isolates in Ukraine. PCV2 genomes were analyzed by phylogenetic tree construction and GIS-based mapping to provinces of origin (Oblasts) in Ukraine: Zaporizhzhia, Chernivtsi, Poltava, Volyn, Lugansk, and Chernihiv Oblasts (B genotype, which is typical for Europe); one PCV2 from Poltava Oblast representing a novel introduction (genotype E). Analysis of PCV2 in swine herds and pork products may indicate trade routes for transmission of swine pathogens.

Bacterial swine co-infection: *Salmonella suis* spp. For the first time, a bacterial pathogen of swine (*Salmonella suis* spp.) was sequenced in Ukraine using MinION technology. This bacterial genome (4,790,000 million DNA bases long) was sequenced in depth in under 24 hours by researchers in the lab at IVM (Kyiv, Ukraine) using rapid ligation library preparation and long read nanopore (MinION) technology.

Bacteriological analysis. The *Salmonella* spp. isolate was cultured, and DNA was purified under standard biosafety level (BSL)-2 level bacteriological protocols applied at IECVM and IVM in Ukraine. The original sample source (pork production facility) was identified by the State Institution Kharkiv Oblast Laboratory Center of the Ministry of Health of Ukraine. The strain was isolated from the Sakhnovshchanskyi Rayon of Kharkiv Oblast. Standard bacteriological analyses were conducted to identify and characterize the strain (**Table 6**); however, the precise genotype of the bacteria was elusive.

Table 6. Bacteriological characterization of *Salmonella* spp.

Characteristics/Properties	**Characteristics**	**Indicator**
Morphological Characteristics	Gram-stain microscopy	Gram-negative rods
Culture Characteristics	Growth in Endo's medium	Pink transparent colonies
	Growth in Bismuth Sulphite Agar	Black colonies with metallic luster, black trace after removal
Biochemical Properties	Carbon source	Lactose - negative fermentation Glucose - positive fermentation Hydrogen sulfide - positive (forms)
	Motility in semi-solid agar	Positive
	Urease activity	Negative
	Indole test	Negative
	Citrate utilization	Positive
	Lysin	Positive
	Sorbite	Positive
	MR	Positive
	VP	Negative
Serological Characteristics	ABCDE	«#»
	O-antigen	O61«#»; O8«#»
	H-antigen	H I ph. – eh «#», h «#» H II ph. – enz15«#», z15«#»

Bacterial DNA isolation and MinION sequencing. Bacterial DNA isolation for whole genome sequencing was performed using DNeasy UltraClean Microbial Kit (QIAGEN). DNA library preparation by RAD004 (Rapid) kit and MinION sequencing followed (**Fig. 10**). The DNA sequencing of purified bacterial DNA was very efficient, exhibiting high occupancy of nanopores (48% at any given time, **Fig. 11**).



Figure 10. Sequencing bacterial swine co-infecting pathogens on MinION in Ukraine. Dr Vasyl Arafiev (IECVM; Kharkiv, Ukraine) works with a flow cell under the supervision of SMEs (*left*) and observes sequencing runs (*right*) at IVM (Kyiv, Ukraine).

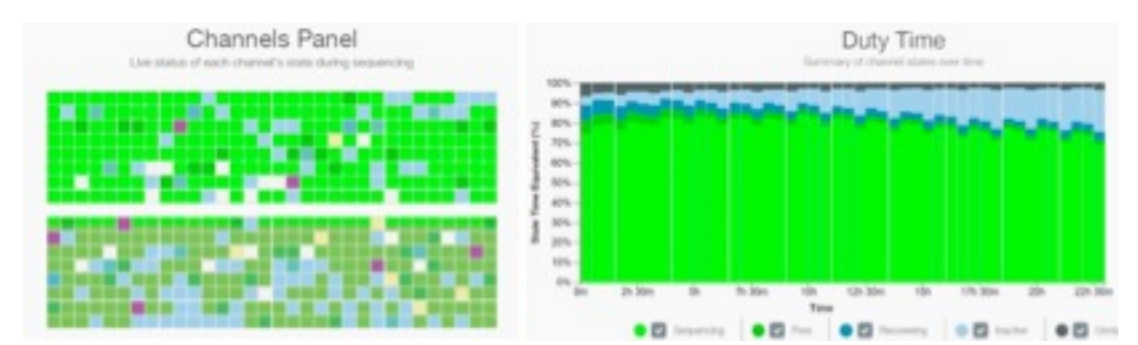


Figure 11. Efficient MinION sequencing of Salmonella spp. MinION device software (MinKNOW) indicated highly efficient sequencing (bright green pores, *left*); and high occupancy over almost 24 hours of sequencing (*right*).

Genome annotation and pathotyping. The whole genome of the bacteria was assembled from basecalled raw data (using a Guppy/Racon/Flye software pipeline developed at UAF), assembling a 4,790,000 million DNA base pair long circular draft genome (**Fig. 12**). The bacterial draft consensus genome was analyzed using the NCBI PATRIC server (https://patricbrc.org/) for genome annotation, analysis of virulence and antibiotic resistance genes, and pathotyping. The 4.7Mbp draft genome contained the complete set of *Salmonella* spp. family genes (**Fig. 12**), as well as antimicrobial resistance marker genes (**Table 7**). Pathotyping suggested an evolutionary branch for this isolate

closely related to the swine- and human-infectious *Salmonella* strains, *S. typhi*, *S. typhiumurium*, and *S. paratyphii*; however, precise pathotyping (serovar) identification is ongoing and requires deeper phylogenetic and virulence factor analyses. A second isolate is also under investigation for independent nanopore sequencing at IECVM (Kharkiv, Ukraine). A manuscript describing this work is in preparation (Arafaev, V., Solodiankin, O., *et al. Isolation and genome analysis Salmonella suis from a swine-product market in Ukraine*).

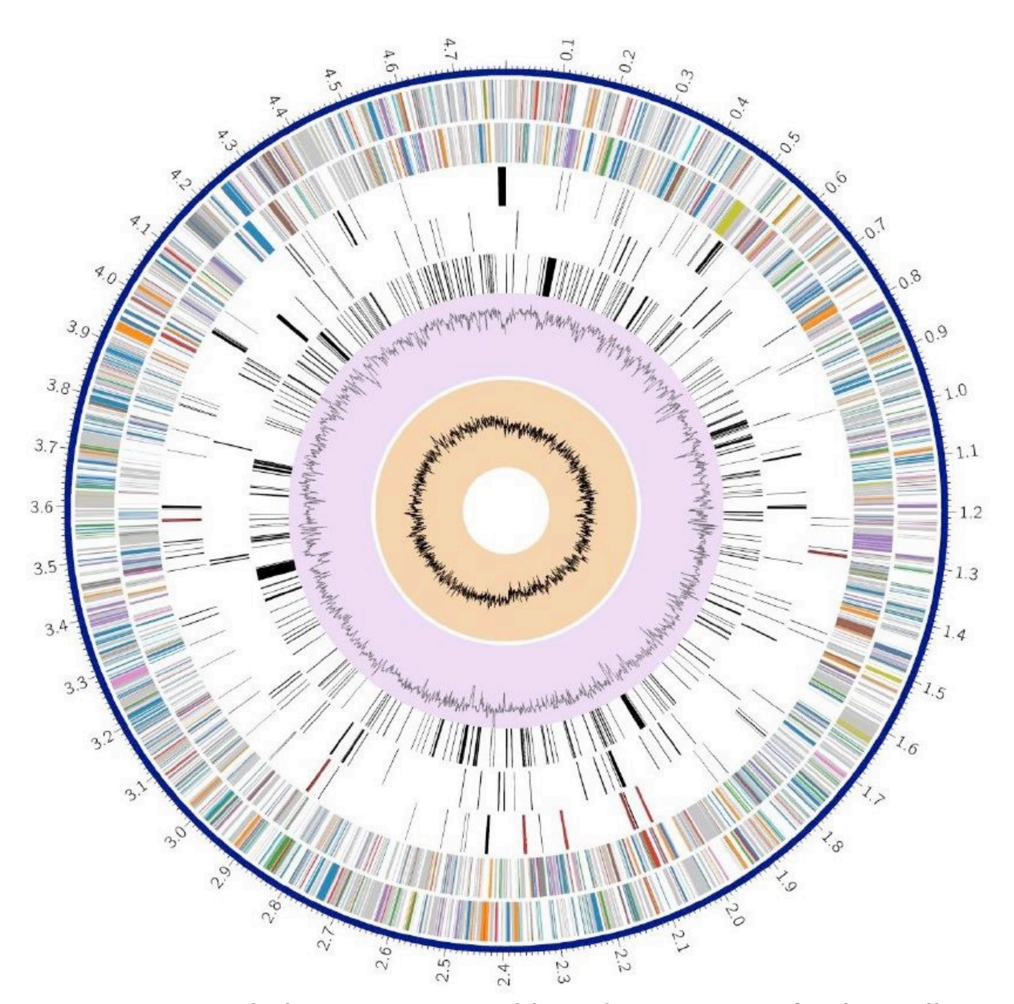


Figure 12. Whole genome assembly and annotation of *Salmonella suis* spp. isolated as co-infection in swine in Ukraine. A 4.7Mbp circular draft genome was assembled and annotated by the NCBI PATRIC (https://patricbrc.org/) bioinformatics server.

Implications of* Salmonella spp. *co-infection in swine in Ukraine. The consequences of salmonellosis in swine and its potential effects on animal susceptibility to ASF is unknown but under investigation. In addition, ASF control and pork food safety measures may coincide, particularly in black market sales of pork products in regions affected by ASF outbreaks.

Table 7. Antimicrobial resistance genes in *Salmonella suis* spp. isolate.

AMR Mechanism	Genes
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	AAC(6')-Ic,f,g,h,j,k,l,r-z
Antibiotic resistance gene cluster,cassette,or operon	MarA, MarB, MarR
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target protection protein	BcrC
Efflux pump conferring antibiotic resistance	AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , EmrAB-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, MexPQ-OpmE, OprM/OprM family, SugE, TolC/OpmH
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Regulator modulating expression of antibiotic resistance genes	AcrAB-TolC, EmrAB-TolC, H-NS, OxyR

Future directions.

Using ASF genome data for ongoing phylogenetics-based epidemiological risk assessment. In summary, research activity focused on MinION sequencing and understanding ASFV genomes. With this methodology, ASF control in Ukraine and the region can include a sequencing component for tracking emerging hot spots, pork trade routes, and potential vectors of ASF spread. These data can be combined with understanding of swine co-infecting pathogens for ASF outbreak tracing, detection, and control. Bioinformatics analysis of genomes made it possible to identify changes in the viral genome that will be characteristic for particular clusters of infection, which would allow for tracking the origin and spread of the virus. Identification of character changes in MGF pathogenicity genes in the ASFV genome, in combination with epidemiological data, will provide an opportunity to better understand the mechanisms of spread and vectors of virus transfer.

Understanding ASFV virulence and informing vaccine design. Understanding the molecular basis of pathogenicity of circulating ASFV strains can contribute to understanding patterns of infection, severity, and control measures. ASFV is a large, enveloped, double-stranded DNA virus that enters the cell by macropinocytosis and a clathrin-dependent mechanism. ASFV is able to interfere with various cellular signaling pathways resulting in immunomodulation, thus making the development of an efficacious vaccine very challenging. Inactivated preparations of ASFV do not confer protection, and the role of antibodies in protection remains unclear. The use of live-attenuated vaccines, although rendering suitable levels of protection, presents difficulties due to safety and side effects in the vaccinated animals. Several ASFV proteins have been reported to induce neutralizing antibodies in immunized pigs, and vaccination strategies based on DNA vaccines and recombinant proteins have also been explored. however, without success. New approaches for creating vaccines can benefit

from current ASFV sequence analyses, such as the viruses sequenced in this study, to help to create an effective vaccine to control ASF.

Summary of co-infections. Like AFSV, swine pathogens are diversely spread across Ukraine, indicating a risk of pathogen spread through a lively swine trade and highlighting the application of PCR diagnostics and MinION sequencing to implement new biosafety measures.

A One Health network of pathogen genomicists in Ukraine and the region. Scientists in Ukraine and regional collaborators (in Poland, Georgia, Armenia, and Moldova), with US SMEs at the University of Alaska, have developed plans for continuation of collaborative work in ASF control, swine co-infections, and other pathogens that impact the health of livestock, wildlife, and humans. Follow on bioinformatics analyses and conference calls with partners will be pursued, in addition to discussions conducted during the project's close-out workshop series. Through these efforts, final consensus genomes will be assembled. A manuscript(s) is in preparation for collaborative publication of scientific results concerning ASFV and co-infections in swine, including bacteriological and epidemiological analyses.

Summary of Task 1.1 and 1.2 project results.

- Ukrainian and regional partner scientists developed and learned genome sequencing protocols using MinION, and advanced bioinformatics analysis, for understanding ASFV and co-infecting pathogens.
- Fourteen (14) ASFV full genomes were sequenced. One ASFV strain from a domestic pig, outbreak #131 (DAN isolated from Kyiv oblast, November 2016), was published and the genome sequence deposited in NCBI GenBank.
- ASF outbreaks were traced in Ukraine by phylogenetic and GIS-based epidemiological analyses, showing two major clusters of infectious spread.
- Viral and bacterial swine co-infections in Ukraine were sequenced using MinION, including PCV2 and *Salmonella suis* spp. which were analyzed using bioinformatics and phylogenetics methodology.
- Ukrainian and regional partner scientists were engaged in training on nanopore sequencing, advanced bioinformatics analysis, viral genome annotation, and phylogenetics to understand ASF and other outbreaks.
- Scientific manuscript writing to report results was also pursued.

Aim 2. Undertake investigations into the epidemiology of ASF in Ukraine to understand exposure, incidence, and prevalence for mapping the disease.

- ***Task 2.1:*** Estimate the roles of environmental risk factors on incidence, persistence, and geographic distribution of ASF outbreaks
- ***Task 2.2:*** Understand ASFV exposure and prevalence in ASF outbreak zones by serological surveillance of domestic pigs and wild boar.

Results and Discussion on Tasks 2.1 and 2.2:

Epidemiological analyses of ASF outbreaks. A detailed analysis of epidemiological data on ASF outbreaks was conducted. In UP-9 OY1, the project's epidemiology group carried out work on analysis of characteristics and identification of risk factors of the ASF outbreaks in the country. All Ukraine ASF outbreaks in 2019 were tracked (**Table 8**), as were outbreaks in the first quarter of 2020 (**Table 9**)**.** From 01 January-31 March 2020, 6 laboratory-confirmed (ASFV DNA detected) ASF outbreaks were registered, and the geographic origins encompassed 4 Oblasts (**Table 9**). Additionally, there was a two-fold decrease in the number of registered cases of ASF compared to the same period in 2019, but two outbreaks in wild boar were registered in Chernihiv Oblast, where outbreaks of ASF were not registered during 2019. ASF outbreaks appear to emerge sporadically, and the underlying factors governing emergence remain elusive. Thus, to understand relative contributions of geographic factors, domestic and wild vectors, and seasonality, a more detailed spatio-temporal modeling approach was employed. In addition, serological analysis of ASF exposure was undertaken.

Serological analyses of ASFV exposure. To understand ASFV exposure and prevalence in outbreak zones by serological surveillance of domestic pigs and wild boar, 1260 samples of sera were collected from different Oblasts: 691 from recent domestic pig outbreaks and 1043 from hunted wild boar. Sera were then tested for the presence of antibodies against ASFV by using a commercial indirect ELISA kit (ID Screen African Swine Fever Indirect / Screening Test), which is designed to detect antibodies against ASFV in porcine serum, plasma, or blood filter paper samples using three recombinant proteins (P32, P62, and P72) as ASFV bait antigens. ELISA results are presented in **Table 10**.

Table 8. Geographic locations of ASF outbreaks in Ukraine, 01 January – 31 December 2019.

№	Oblast	ASF Outbreaks				
		Domestic Pigs			Wild Boar	Total Number of ASF Outbreaks in Each Oblast
		Backyard	Farm	Infected Object		
1	Dnipropetrovsk	3				3
2	Donetsk	4			1	5
3	Mykolaiv	6		2		8
4	Kyiv	2				2
5	Kharkiv	1		1		2
6	Ternopil	2		1		3
7	Chernivtsi				3	3
8	Volyn				2	2
9	Zaporizhzhia	1	1		1	3
10	Khmelnytskyi		1			1
11	Kirovohrad	2	1		1	4
12	Vinnytsia	1	2			3
13	Sumy	1				1
14	Kherson	2	1			3
15	Poltava		3	1		4
16	Lviv		1			1
17	Zhytomyr	1	1		1	3
18	Odesa			1	1	2
Total ASF Outbreaks in Ukraine, 01 January – 31 December 2019		43 (Domestic Pigs)			10 (Wild Boar)	**53**

Table 9. Geographic locations of ASF outbreaks in Ukraine, 01 January – 31 March 2020.

№	Oblast	ASF Outbreaks				
		Domestic Pigs			Wild Boar	Total Number of ASF Outbreaks in Each Oblast
		Backyard	Farm	Infected Object		
1	Chernihiv				2	2
2	Rivne			1		1
3	Vinnytsia	1	1			2
4	Volyn				1	1
Total ASF outbreaks in Ukraine, 01 January – 31 March 2020		3 (Domestic Pigs)			3 (Wild Boar)	**6**

Table 10. Serological analysis of ASF exposure in Ukraine.

Oblast	Domestic pigs		Wild Boar	
	# of Samples	# of Positive samples	# of Samples	# of Positive Samples
Vinnytsia	25	0	264	1
Donetsk			3	0
Zhytomyr	35	0	89	2
Zakarpattia			17	0
Ivano-Frankivsk			30	0
Lviv	15	0	12	0
Mykolaiv			54	0
Odesa	320	0	54	1
Poltava	60	0	88	2
Rivne			78	0
Sumy	350	3	57	0
Ternopil			47	0
Kherson	150	0	0	0
Khmelnytskyi	15	0	0	0
Cherkasy			22	0
Chernivtsi	5	0	160	0
Chernihiv	66	0	68	0
TOTAL:	**691**	**3 (+)**	**1043**	**6 (+)**

Serological interpretation. Three (3) domestic pigs from Sumy (0.43% seroprevalence) and 6 wild boar (0.57% seroprevalence) from Vinnytsya (1), Zhytomyr (2), Odesa (1), and Poltava (2) were seropositive. All seropositive samples were tested by PCR with negative results, suggesting prior exposure to ASFV and survival of the seroconverted animal. These data are lower than 1-5% seroprevalence found in serological studies of ASF exposure in wild boar in Poland (NVRI, unpublished data). Additional analyses are required to further understand these findings; however, such comparative studies cannot be pursued until COVID-19-related restrictions are lifted.

Spatio-temporal modeling analysis of ASF outbreaks in Ukraine. GIS-based epidemiological analyses of ASF outbreaks were undertaken to discover patterns in pathogen spread in Ukraine. The methods developed are potentially of use by regional partners with ongoing ASF outbreaks (Ukraine, Poland, Moldova, Romania, and the Baltic countries). The data sources used for these analyses included: 1) http://www.asf.vet.ua/ website, which is supported by DNDILVSE, and 2) http://empres-i.fao.org/eipws3g/ Global Animal Disease Information System (EMPRES-i) supported by FAO. The graphs and maps were built in R (free software environment for statistical computing and graphics, https://www.r-project.org/) and ESRI ArcGIS 10.4 software.

Descriptive statistics and temporal distribution of the outbreaks. ASF started to spread throughout the territory of Ukraine in 2014, and by 2017, outbreaks were reported in all Oblasts of the country (**Fig. 13**). However, the annual number of outbreaks in both domestic pigs and wild boar have been decreasing since 2018, with outbreaks reported predominantly in the former (**Table 11, Fig. 14).** As of 02 April 2020, 8 ASF outbreaks have been reported, fewer than the same period in 2019 (12 outbreaks), and all outbreaks in domestic pigs were reported in western and central Ukraine (Rivne, Vinnytsia, and Kirovohrad Oblasts) and southern Ukraine (Kherson Oblast) (**Fig. 15**). The outbreaks emerged only on the right bank of the Dnieper river except one in southern Ukraine. Two outbreaks occurred in backyard farms, two at industrial farms, and one was a carcass. Only 3 outbreaks were reported in wild boar during this time period, one in Volyn Oblast in the western part of Ukraine near the borders with Poland and Belarus, and two in the north (Chernihiv Oblast) near the border with Belarus (**Fig. 15**). The temporal distribution of the outbreaks in wild boar peaked later than in domestic swine (**Fig. 14**). Seasonality data indicated the highest number of outbreaks in wild boar in winter months and in July. No cases in wild boar were reported in September. In domestic pigs, the highest number of outbreaks were detected in July, August, and October. The smallest number of outbreaks in both species were reported during springtime (**Figs. 16 – 17**).

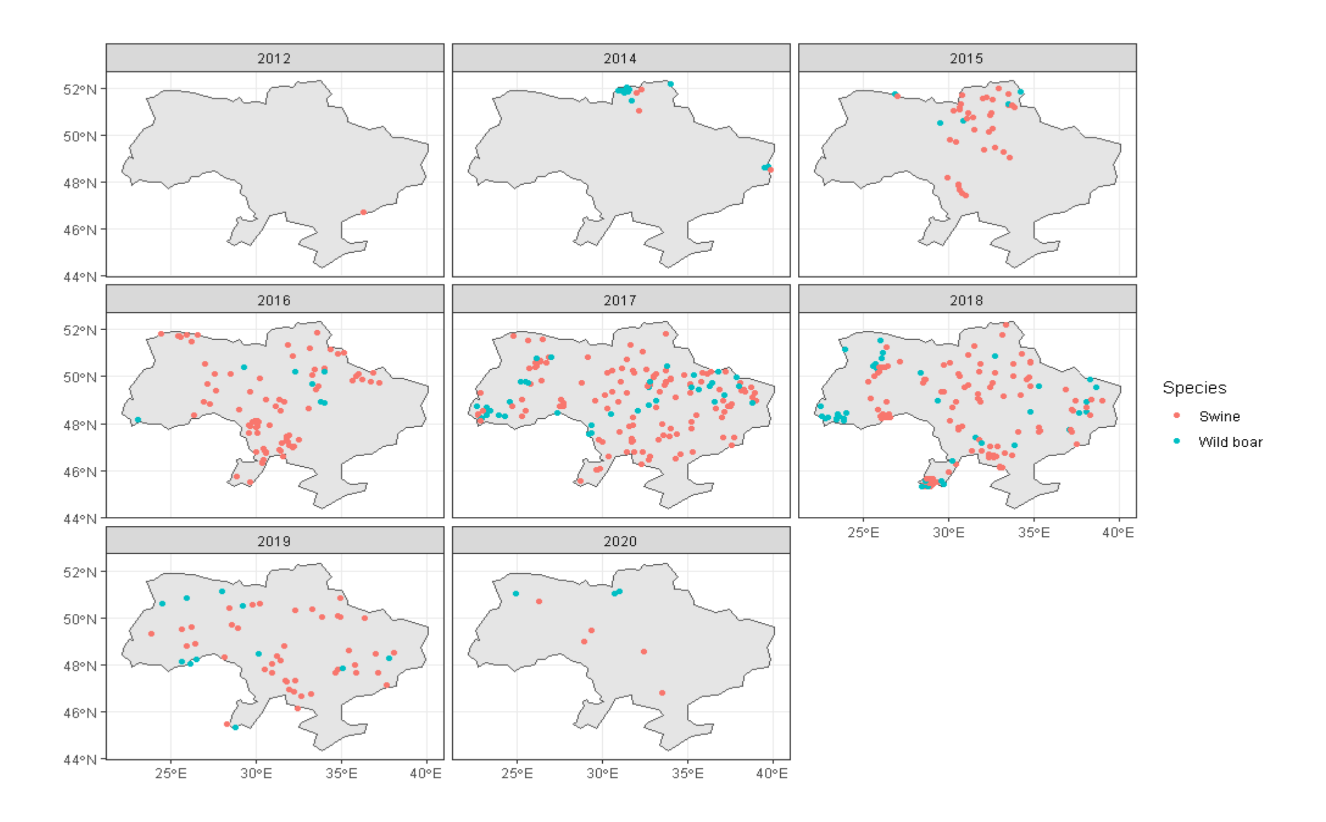


Figure 13. Spatial distribution of ASF outbreaks in Ukraine. Domestic swine (red dots) and wild boar (blue dots) indicated for 2012-2020.

Table 11. Annual number of ASF outbreaks in Ukraine by subspecies.

	2012	2013	2014	2015	2016	2017	2018	2019	2020
Domestic Pigs	1	0	4	35	84	125	105	42	5
Wild Boar	0	0	12	5	7	38	40	11	3

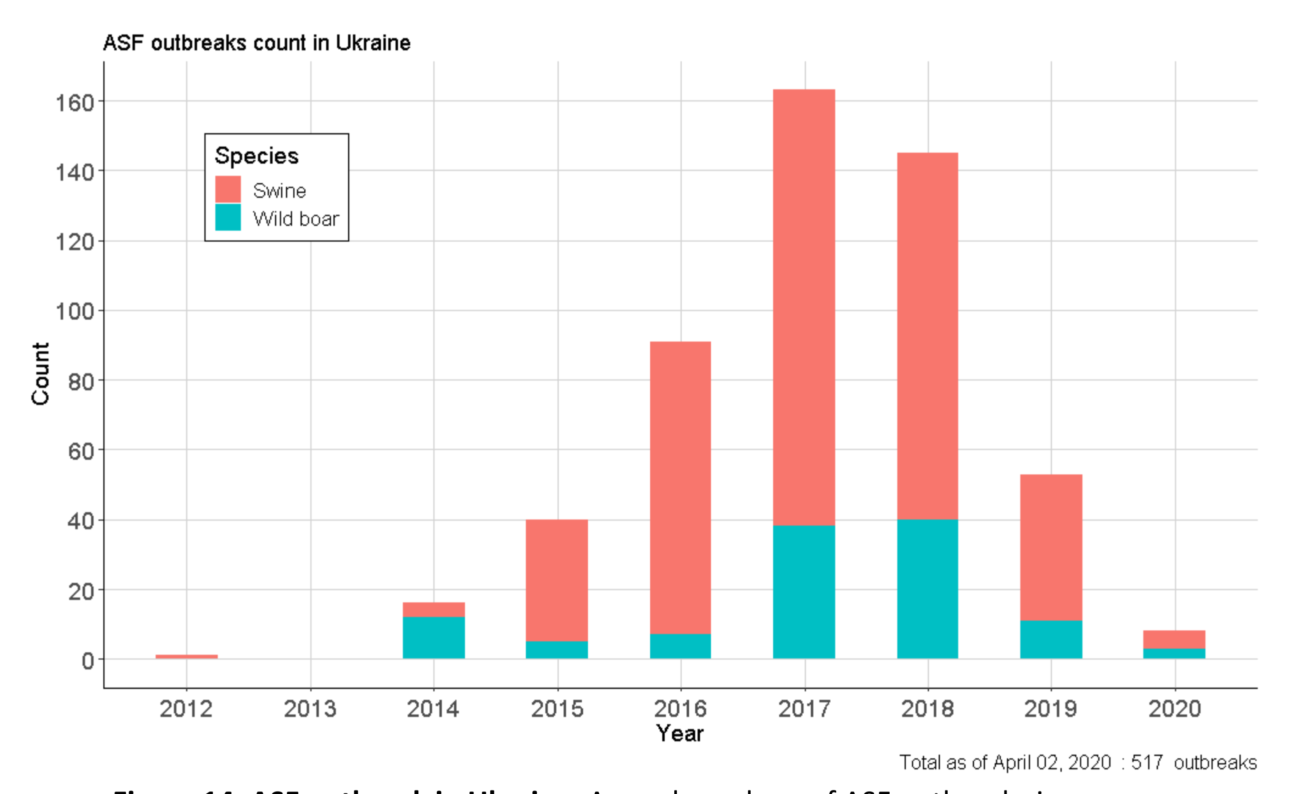


Figure 14. ASF outbreak in Ukraine. Annual numbers of ASF outbreaks in domestic pigs (red) and wild boar (blue).

Spatial and temporal analysis of ASF outbreak trends in Ukraine. To describe the dynamics of the spatial distribution of ASF outbreaks, *Trend surface* analysis was used. This is a least squares regression method used to examine coarse-scale spatio-temporal patterns in the cumulative outbreak data (**Figs. 13 – 17**). For the analysis, the date of ASF outbreak was treated as the dependent variable in a regression model, which uses spatial coordinates as its independent variables.

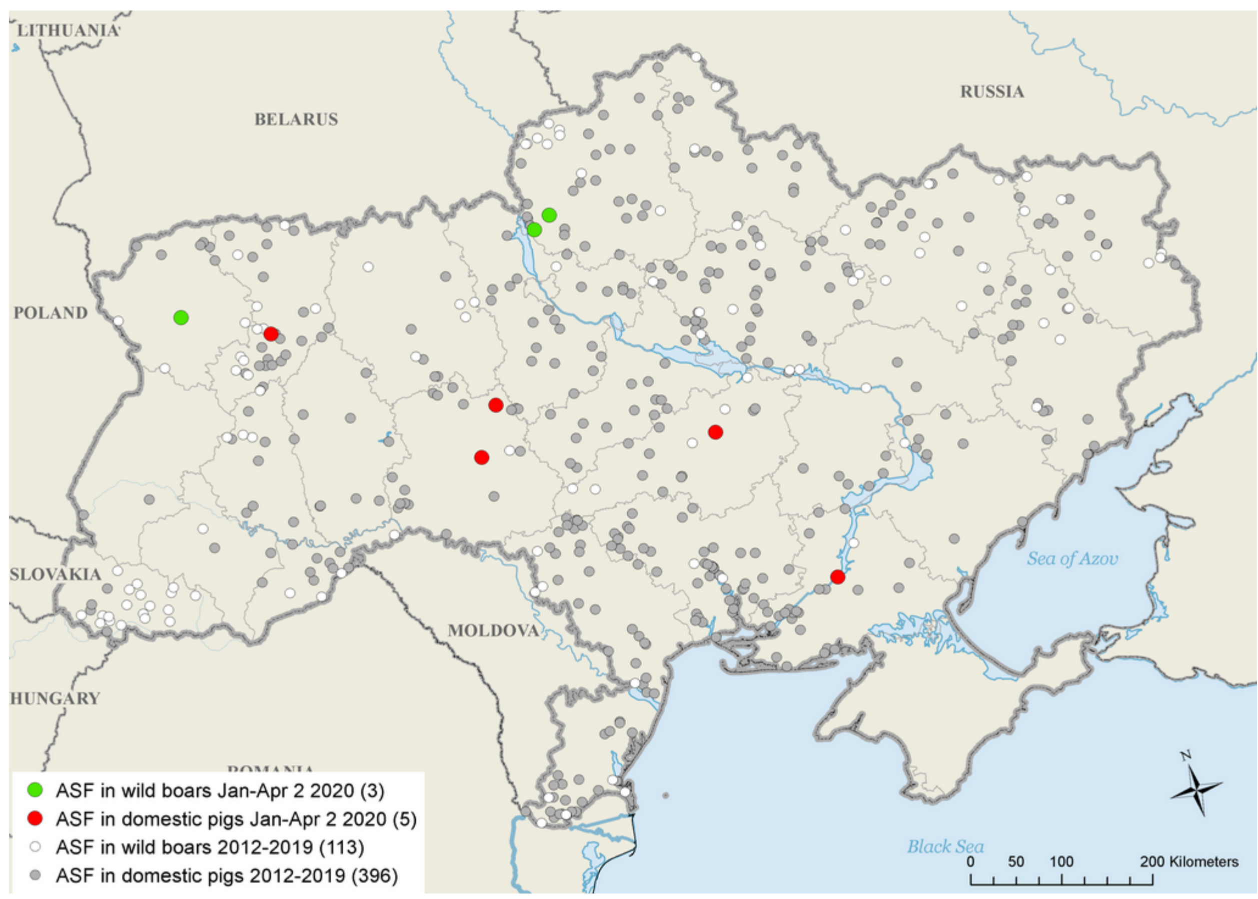


Figure 15. Geographic distribution of ASF outbreaks in Ukraine. ASF outbreaks in wild boar (green) and domestic pigs (red) in 2020 (as of 02 April 2020).

Figure 16. Outbreak dynamics of ASF in Ukraine. Epidemiological curve in domestic pigs (red line) and wild boar (blue line) by month since 2014. Dashed lines describing temporal trend were calculated with LOESS smoothing.

Figure 17. Outbreak seasonality of ASF in Ukraine. Monthly distribution of ASF outbreaks in both domestic pigs (red) and wild boar (blue).

Results: illustrating ASF spread geographically in Ukraine. Analysis was conducted using data from the asf.vet.ua website for the country's outbreaks from 2014-2017 (**Fig. 18**). A second analysis was performed with the EMPRES-i dataset, which included outbreaks starting from January 2012 (**Figs. 19-20**). For these analyses, outbreaks inside 150 km buffer around the country's borders were investigated. Analysis of both datasets demonstrated the distribution of ASF outbreaks from northern to southern Ukraine. ASF then spread west, southwest, and east, risking transboundary transmission to neighboring countries (**Fig. 20**).

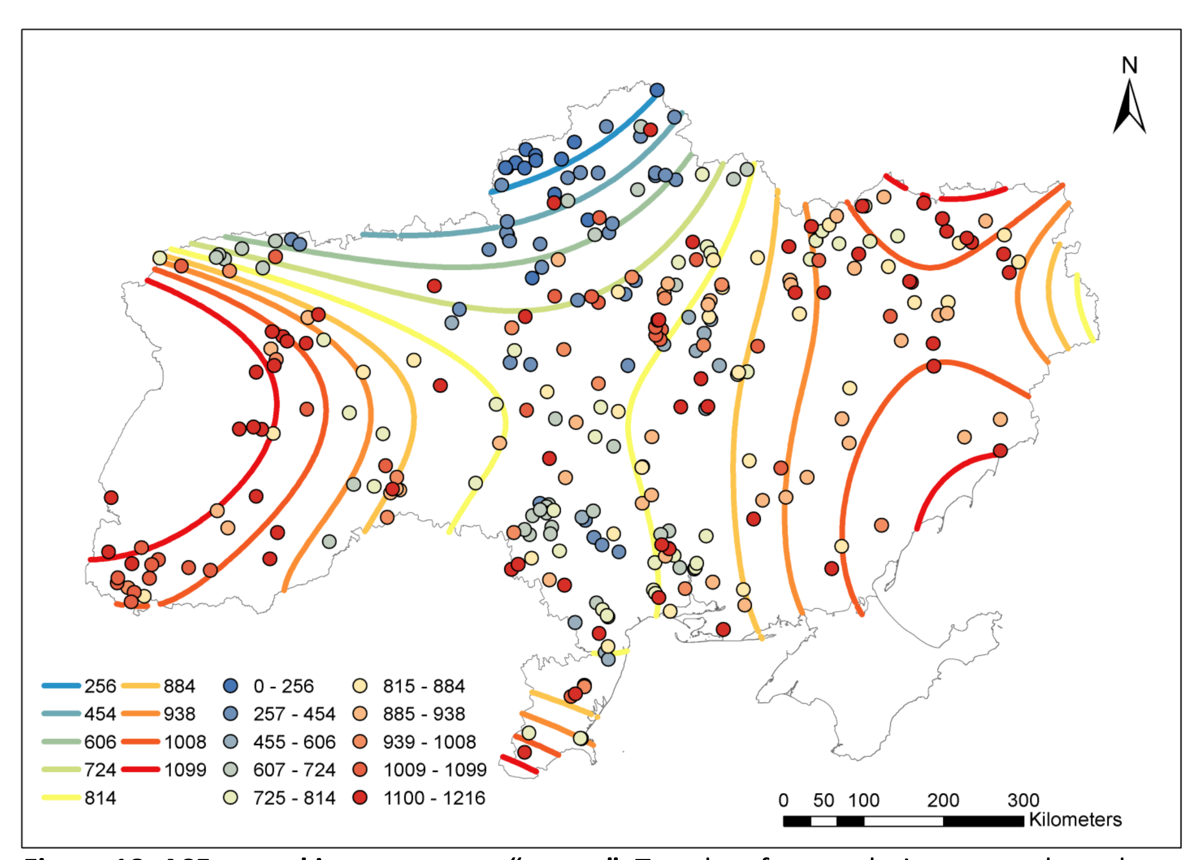


Figure 18. ASF spread in contours or "waves". Trend surface analysis was conducted as described in the text. Colored contours show the average diffusion of ASF outbreaks in space and time (days from the first outbreak, 2014-2017). Earliest (blue) and latest (red) outbreaks are depicted, with dots representing all ASF outbreaks.

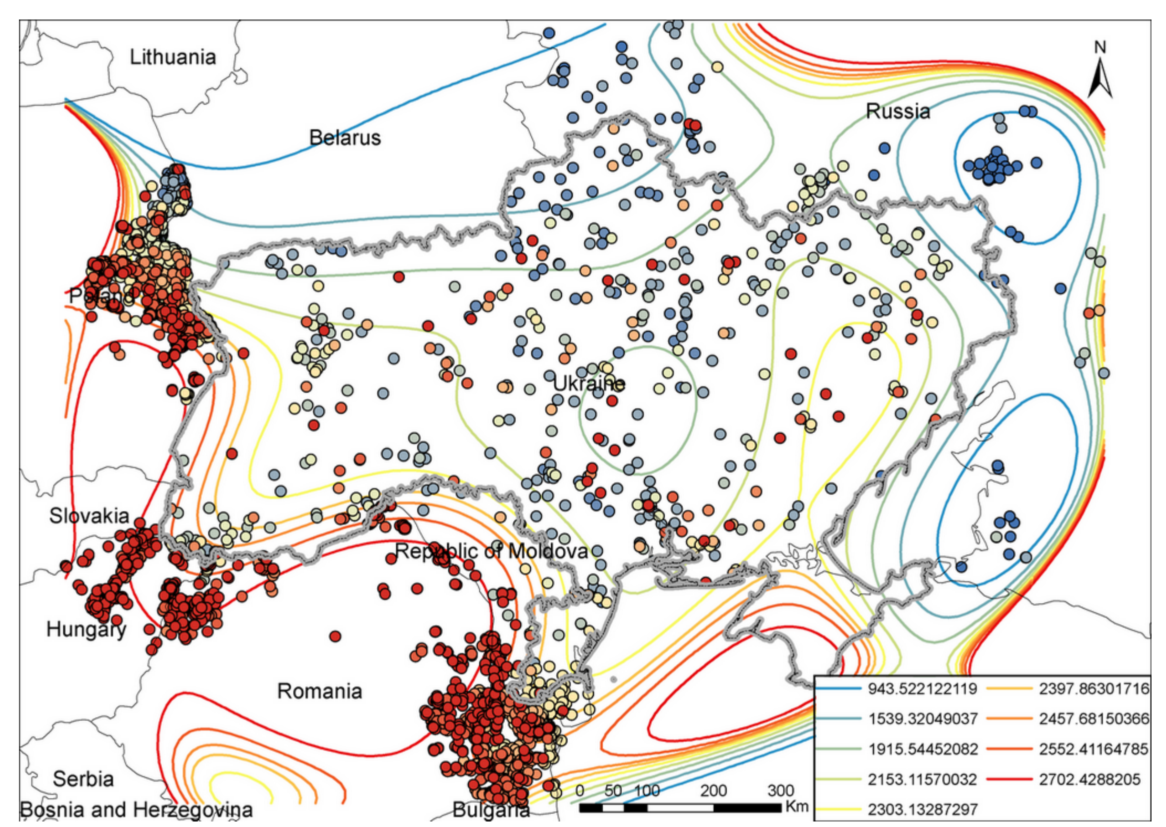


Figure 19. Diffusion of ASF outbreaks in contours or “waves” in Ukraine that are closely linked to transborder spread of ASF in the region. Trend surface analysis was conducted as described in the text. Colored contours show average diffusion of ASF outbreaks in space and time (days from the first outbreak, 2012 onward). Earliest (blue) and latest (red) outbreaks are presented.

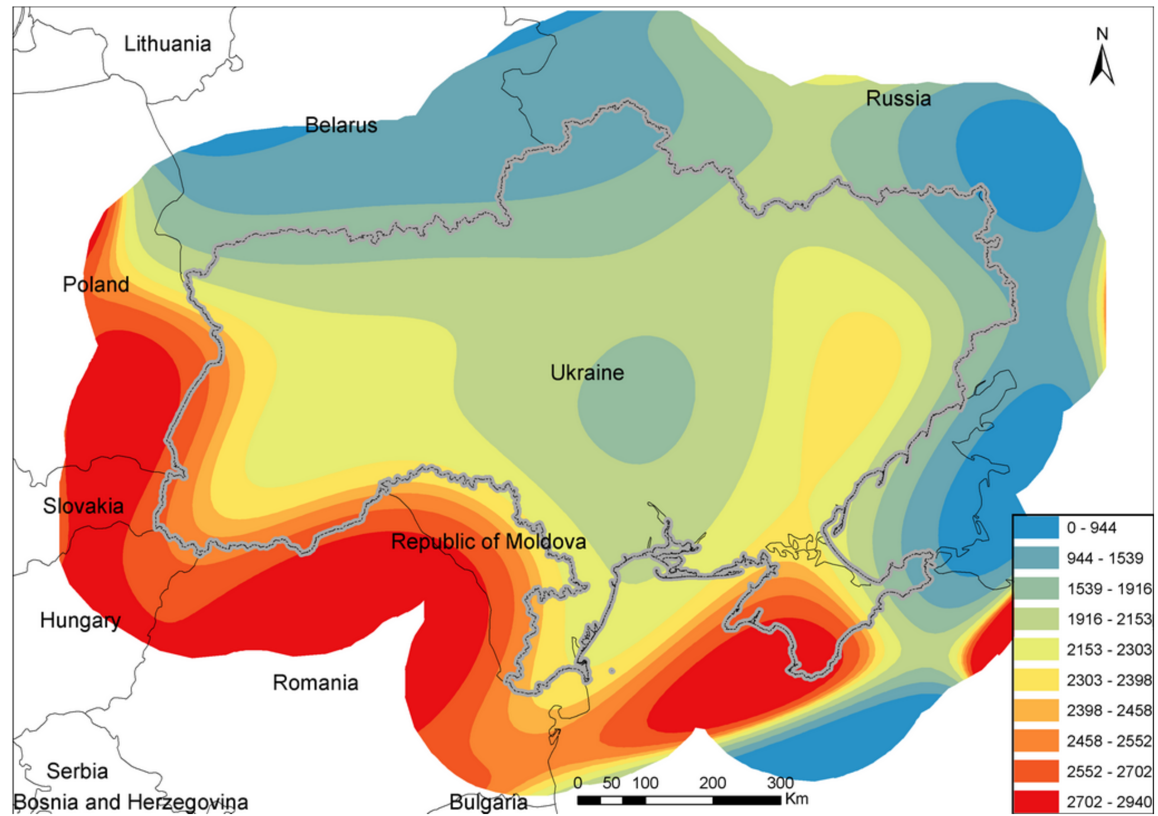


Figure 20. Risk of ASF outbreaks in contours or "waves" in Ukraine that are closely linked to transborder spread of ASF in the region. Trend surface analysis was conducted as described in text. Colored contours show the average diffusion of ASF outbreaks in space and time (days from the first outbreak, 2012 onward). Earliest (blue) and latest (red) outbreaks are presented.

***Heatmap analysis of ASF outbreaks in Ukraine*.** GIS-based heatmap analyses provided a predictive estimate of ASF outbreak density as the infection spread across the country from 2014-2020. The kernel density estimation (KDE) method was used to build heatmaps of ASF outbreaks. For this analysis, the density function available in spatstat package in R was used. In 2014-2020, the largest cumulative spatial intensity of ASF outbreaks was observed in Poltava Oblast in eastern Ukraine, Odesa and Mykolaiv Oblasts in the south, and Rivne and Zakarpattia Oblasts in the west (**Fig. 21**). In domestic pigs, the highest density was located in Poltava Oblast in eastern Ukraine, Odesa and Mykolaiv Oblasts in the south, and Rivne Oblast in the west (**Fig. 22**). In wild boar, the highest density was located in western and northern Ukraine and in Odesa Oblast near the border with Romania (**Fig. 23**). This coincides with the territories in Ukraine where wild boar are most common. Average density of outbreaks per square kilometer in domestic swine were 3.5 times higher than in wild boar. Several new outbreaks in 2020 occurred inside the major hot spots detected between 2012-2020, suggesting annual persistence of ASFV in vectors (**Fig. 24**).

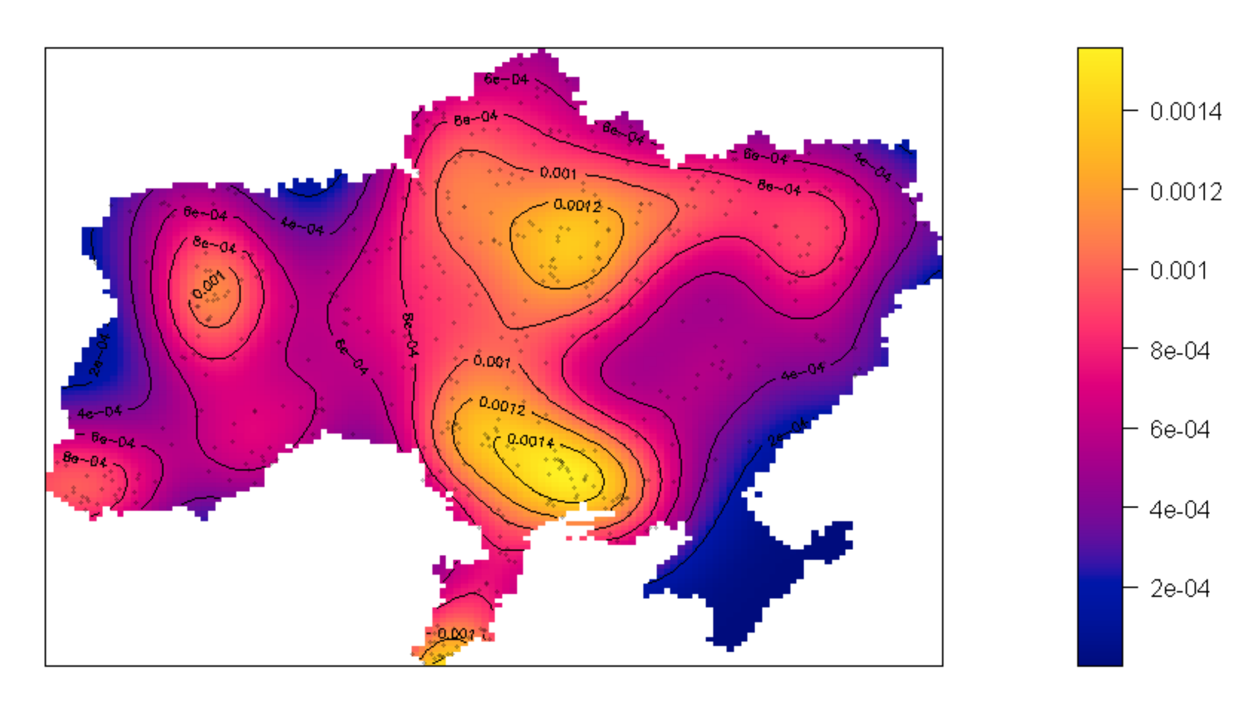


Figure 21. Heatmap analysis of ASF outbreak density. Total density of ASF outbreaks per square kilometer 2014-2020 in both wild boar and domestic swine by KDE methods (see text).

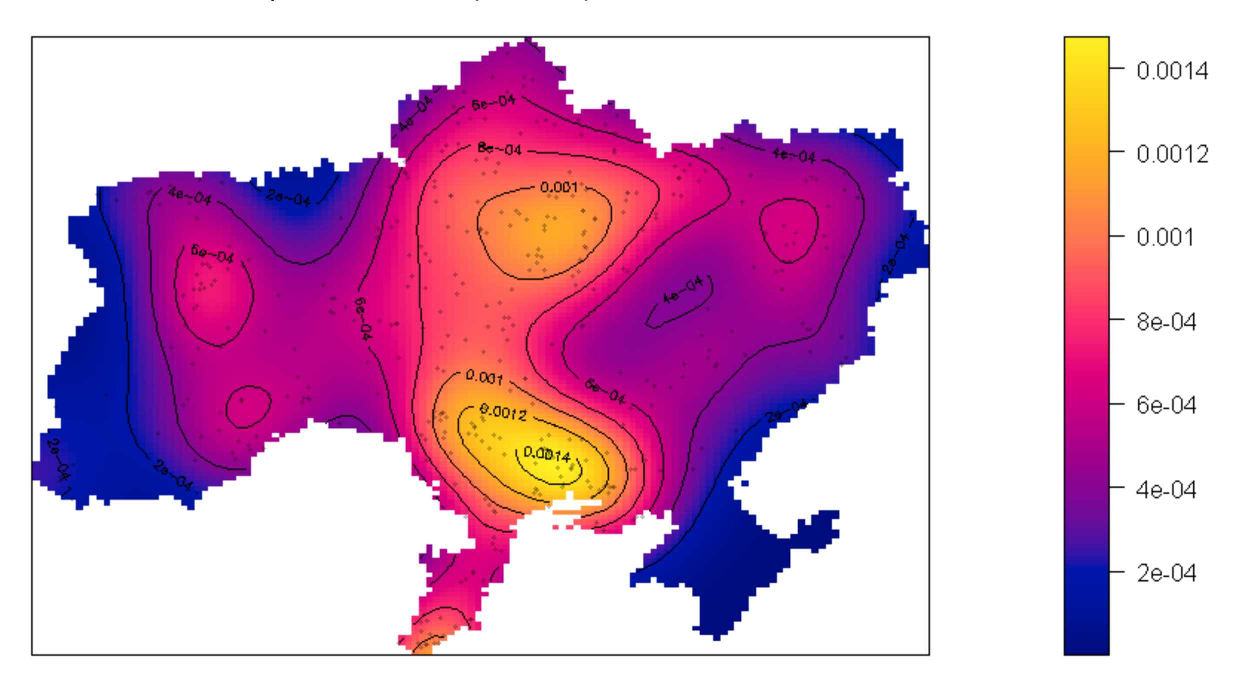


Figure 22. Heatmap analysis of ASF outbreak density in domestic swine. Density of ASF outbreaks in domestic pigs per square kilometer 2014-2020.

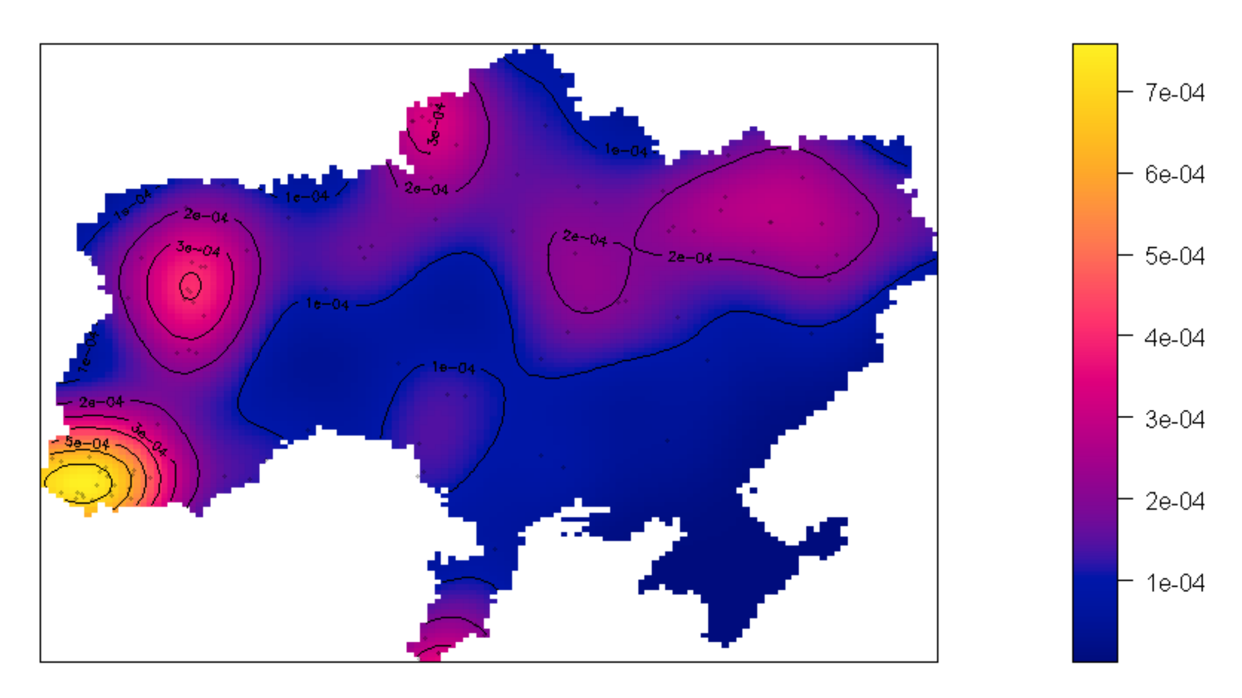


Figure 23. Heatmap analysis of ASF outbreak density in wild boar. Density of ASF outbreaks in wild boar per square kilometer 2014-2020.

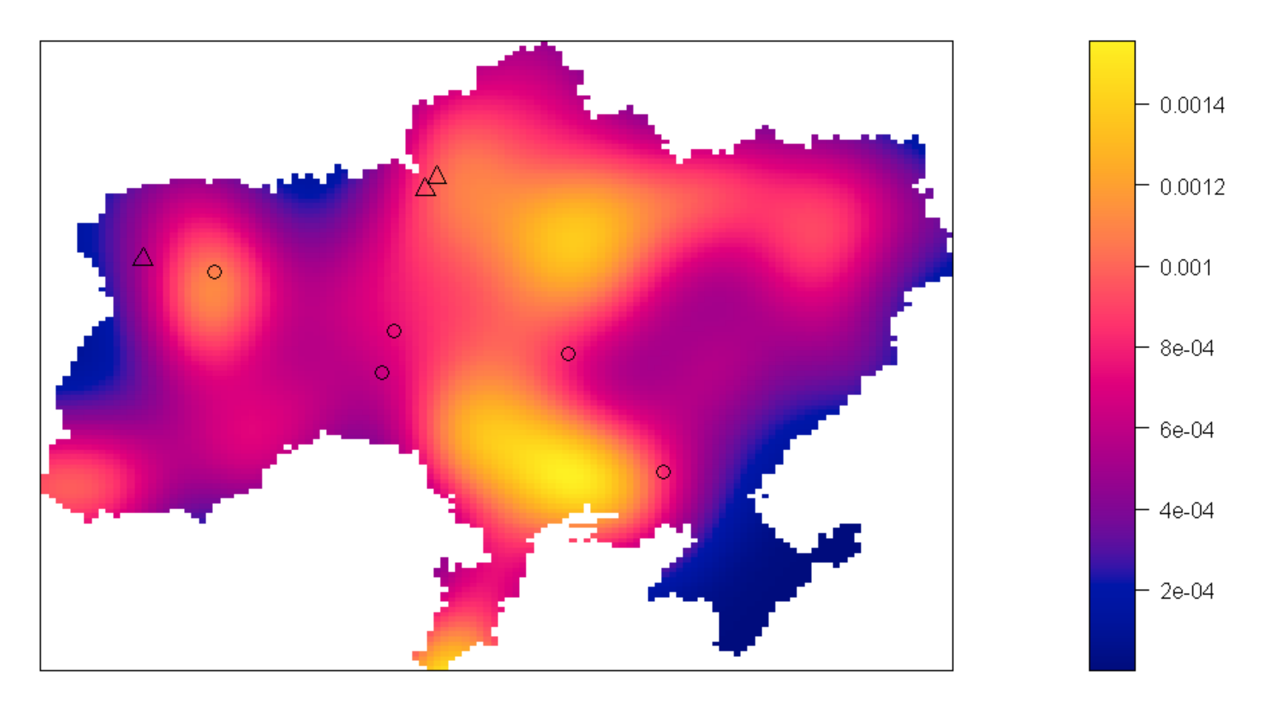


Figure 24. Heatmap analysis of ASF outbreak density with current (2020) outbreaks located near historical ASF outbreak regions. Density of ASF outbreaks per square kilometer 2014-2020. Outbreaks in 2020 in domestic pigs (circles) and in wild boar (triangles).

Space-time scan statistic (SatScan) analysis. To detect spatio-temporal clusters of ASF outbreaks, a space-time permutation model was implemented using SatScan software. Space-time clusters presented in **Fig. 25** and **Table 12** included outbreaks in both domestic pigs and wild boar.

Multi-distance spatial cluster analysis. To determine whether ASF outbreaks exhibit statistically significant clustering or dispersion over a range of spatial scales, a Multi-Distance Spatial Cluster Analysis (Ripleys K) was used. Application of this method was conducted using the Kest function in the spatstat package in R. In summary, outbreaks in wild boar were more clustered than in domestic pigs at distances up to 180 km and peaked sooner and dropped off more rapidly than for domestic pigs. This suggests that many outbreaks in wild boar tend to occur in tighter clusters while clusters for domestic pigs are more spread out. The distribution of the outbreaks in domestic pigs shows statistically significant clustering within 15-75 km distance around outbreaks in wild boar. This suggests that groups of wild boar are a key vector for long range dispersal of ASF and spread to domestic swine farms in Ukraine, as well as in similar mixed forest and agricultural biomes in the region.

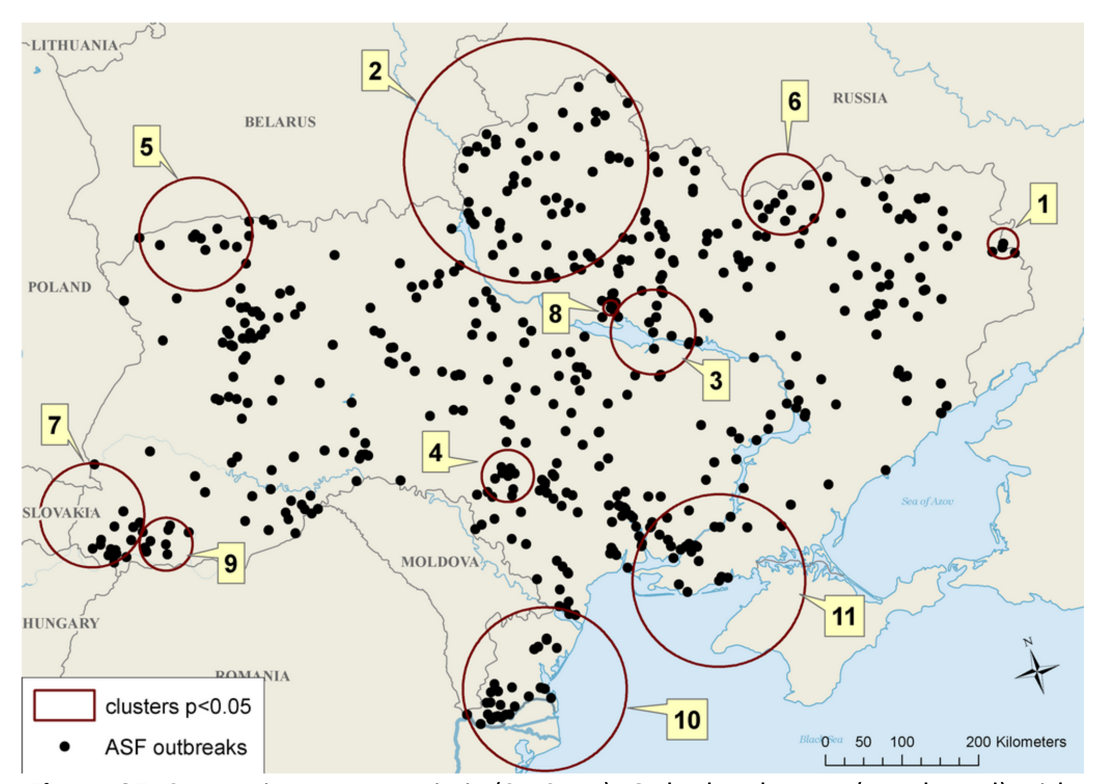


Figure 25. Space-time scan statistic (SatScan). Only the clusters (numbered) with statistical significance level p<0.05 are presented.The clusters are ordered by start day of their occurence. Dots represent ASF outbreaks both inside and outside clusters.

Table 12. Space-time cluster statistics of ASF spread in Ukraine.

Cluster Number	Radius, km	Start Day	End Day	# of Outbreaks Observed	Expected	p Value
1	20.02369973	1/6/2014	2/16/2014	4	0.031434	0.00084
2	161.1435726	8/31/2014	10/22/2015	35	4.255403	1E-17
3	55.77323598	12/17/2015	3/16/2016	7	0.188605	1.87E-05
4	34.28746795	7/26/2016	8/18/2016	10	0.664047	4.22E-05
5	74.63692286	8/18/2016	8/26/2016	7	0.259332	0.000265
6	53.22191779	8/30/2016	10/7/2016	6	0.237721	0.007528
7	68.65564116	5/30/2017	9/27/2017	11	1.257367	0.003757
8	9.936607728	7/7/2017	7/20/2017	4	0.062868	0.019
9	34.86868767	2/1/2018	2/1/2018	6	0.159136	0.000433
10	107.2721023	6/21/2018	7/27/2018	16	1.644401	1.95E-07
11	113.264899	7/13/2018	11/5/2018	16	2.656189	0.000713

Summary and key threat reduction impacts: Identification of regional risk. Since 2014, ASF spread throughout the territory of Ukraine and in neighboring countries. During UP-9 OY1 workshops, detailed epidemiological mapping analyses led by scientists at IVM and other institutions (SSRILDVSE, IECVM, NVRI, and SMEs) revealed the spatial and temporal spread of ASF in Ukraine. The modeling methods employed clearly illustrated the spread of ASF in Ukraine and the risks to neighboring countries, illuminating the difficulty in controlling ASF. without more detailed knowledge of mechanisms of seasonal persistence, transit, and pathogenesis of ASFV.

To mitigate risk, additional research into the mechanisms of ASF spread is warranted (e.g., more detailed knowledge into seasonal persistence, transit, and pathogenesis of ASFV), including assessment of the risk wild boar serve as a vector across international borders. The genomics-based epidemic tracing (Task 1.1) has contributed to this effort by identifying genomic links between ASF outbreaks, thereby enriching epidemiological modeling data and providing insight to stakeholders and regional partners.

A manuscript describing the team's epidemiological analytical work is in preparation, representing a collaborative analysis with all research groups in UP-9 (Bezyemenni M., *et al*. *Spatio-temporal outbreak model of the spread of African swine fever in Ukraine, 2014-2020*).

Aim 3. Scientific Advancement: Bioinformatics capacity-building and data sharing.

- ***Task 3.1.*** Advance scientific capacity for pathogen genomic analysis through bioinformatics and sequence analysis workshops.
- ***Task 3.2:*** Advance ASF data sharing, reporting, and international collaboration through workshops, communication among institutes in Ukraine and SMEs, and scientific presentations and publications.

Results and Discussion on Tasks 3.1 and 3.2.

Scientific Advancement: ***Ukrainian and regional capacity-building and collaboration in ASFV genomics and epidemiological analysis.*** Throughout UP-9 base period and UP-9 OY1 activities, Ukrainian and regional partner scientists engaged in hands-on research activities centered around building skills in virus genome sequencing using MinION methods, advanced bioinformatics, and GIS-based epidemiological analysis tools. These practical skills were built in the course of the research efforts described above (Tasks 1 and 2), both as exercises and actual research activities during workshops. A diverse group of scientists from 4 institutions in Ukraine (SSRILDVSE, IVM, IECVM, and UAPRI), one in Poland (NVRI), three in Georgia (LMA, Ilia State, and Georgia CDC), and MoA/university partners in Armenia and Moldova directly participated in research activities, building collaborations, expertise, and insight into ASF and other swine outbreaks threatening the region. Project SMEs from the University of Alaska, Metabiota, and others guided, and as appropriate, mentored these scientists. The UP-9 group forms the core of a genomics-based infectious disease threat reduction network for Ukraine and partner countries. It is the recommendation of project SMEs that these skills continue to be fostered to build capacity for infectious disease control (for ASF and other livestock, wildlife, and human pathogens). In addition, development of scientific presentations, manuscripts, and participation in international conferences highlighted the expertise gained by project scientists, as well as the trust build between Ukrainian, regional participants, and project SMEs.

Regional engagement highlights included:

- Collaboration was fostered among regional scientists through invitation to communicate on ASF outbreaks and to develop a regional scientific network aimed at the reduction of the threat of ASF outbreaks and other infectious diseases.
- Scientists included UP-9 researchers from Ukraine (SSRILDVSE, IVM, IECVM, and UAPRI); Poland (NVRI, G. Wozniakowski *et al.*); Georgia (NCDC, M. Ramishvili; MoA/Lab., M. Kokhreidze; ISU, L. Ninua); Armenia (N.Vet.Inst., S. Kharyatan); Moldova (MoA/NFSA, I. Navarascaia); and communication with researchers in Azerbaijan, Turkey, Romania, Estonia, as well as information exchanged with project SMEs at the University of

Alaska (E. Bortz; D. Drown, et al.), Metabiota (C. Lange), Inna Dubchak (JGI), and research colleagues in Germany (FLI), Kansas State University (US), Mount Sinai School of Medicine, and the USDA.

- Priorities were discussed for Ukrainian and regional collaborators to learn advanced genomics-based outbreak diagnostics using nanopore (MinION) sequencing, associated advanced bioinformatics capacity-building, and using pathogen sequence data in epidemiological analyses. The potential of rapid MinION sequencing for identification, pathotyping, and subtyping of novel outbreaks was discussed in workshop research.
- Virtual bioinformatics videoconferencing was conducted between the University of Alaska, Ukraine (SSRILDVSE, IVM, IECVM), and Georgia (NCDC) to discuss advanced bioinformatics approaches for quality analysis of raw MinION sequence data, virus genome annotation, and phylogenetics analysis of ASFV and co-infections.
- Follow-on discussions were held regarding research activities conducted during the aforementioned Workshops and data analysis.
- Development of abstracts, methods descriptions, and data figures for publication of results of UP-9 research were pursued (see Appendix C).

In addition to close scientific engagement among researchers in Ukraine, and connection to the international scientific community and SMEs from University of Alaska (*et al.),* regional partnerships were pursued to build collaborations among scientists working on ASF and other infectious disease control efforts. Project SMEs and lead scientists from Ukraine, particularly IECVM and SSRILDVSE, fostered these regional collaborations through electronic communication, discussion at conferences (ASF STOP, DTRA SPR, ASM Biothreats, ASV, et al.), and invitations to partners. A list of regional collaborating partners was developed (**Table 14**) and can be adopted for future engagements.

Table 14. Regional partner engagements in UP-9 OY1.

Country	Point of Contact	ASF Collaboration Activities
Georgia	**Marina Ramishvili** (Lugar Center (CDC Georgia) m.ramishvili@ncdc.ge **Zura Javakhishvili** (Institute of Ecology, Ilia State University, Georgia; via contact with Levan Ninua, levan.ninua@iliauni.edu.ge **Maka Kokhreidze** (Lab. Min. Agriculture/LMA) Maka.kokhreidze@lma.gov.ge	• UP9 OY1 Overview Brief distributed and electronic communication initiated. • Referral to ISU and LMA institute directors and ASF research staff through PoC listed (in coordination with UP-4 activities in Genomics and Concurrent Infection Workshop, Kharkiv, Ukraine; June 2019). • Discussed data sharing and set up of rapid ASFV genome sequencing protocols in lab at LMA for supporting genotype data to diagnose new ASF cases in Georgia. • Participated in UP-9 OY1 workshops in genomics and epidemiology in Ukraine.
Armenia	**Tigran Markosian** (Armenian National Veterinary Institute, Yerevan, Armenia) tigran79hm@yandex.ru **Satenik Kharatyan** (Senior Scientist, Head of Department Molecular Biology and Serology, NVI) satenik_vet@mail.ru	• UP9 OY1 Overview Brief distributed and electronic communication initiated, following up from contact and in-country discussions by O. Solodiankin (IECVM, Ukraine). • Participated in UP-9 OY1 workshops in genomics and epidemiology in Ukraine.
Estonia	**Arvo Viltrop** (EMÜ Veterinaarmeditsiini ja loomakasvatuse instituut, Tartu, Estonia) Arvo.Viltrop@emu.ee	• UP9 OY1 Overview Brief distributed and electronic communication initiated, following previous contact at ASF epidemiology training school at FLI, organized by ASF-STOP EU Horizon 2020 project.
Romania	**Georgeta Stefan** (U. of Agricultural Sciences and Veterinary Medicine, Bucharest, Romania). getastefan@yahoo.com)	• UP9 OY1 Overview Brief distributed and electronic communication initiated, following up from contact and in-country discussions by O. Solodiankin (IECVM, Ukraine).

Country	Point of Contact	ASF Collaboration Activities
Poland	**G. Wozniakowski** and colleagues (Nat. Vet. Res. Inst./NVRI, Pulawy, Poland)	• Discussed UP-9 OY1 workshops in genomics and epidemiology, sequencing methods, and conducted site visit workshop for ASFV genomics at NVRI. • NVRI collaboration via UAA. Participated in UP-9 OY1 workshops in genomics and epidemiology in Ukraine (Maciej Frant and Natalia Mazur).
Moldova	**Inna Naraevscaia** (National Agency Food Safety, Chisinau, Moldova)	• Discussed UP-9 OY1 workshops in genomics and epidemiology, sequencing methods, and conducted site visit workshop for ASFV genomics at NVRI. • Participated in UP-9 OY1 workshops in genomics and epidemiology in Ukraine.
Other: **Germany** **USA, Singapore, Kenya,** **Turkey** **Azerbaijan**	Sandra Blome and colleagues (Friedrich Loffler Institute/FLI, Germany) Jurgen Richt and Wenjun Ma (Kansas State U., USA); Doug Gladue (USDA); Gavin Smith (Duke-Nat. Univ. Singapore); Ed Okhosh (ILRI, Kenya); Ahmet Deniz, VCCRI, Ankara Shalala Zeynalova, MoA	• Discussion during genomics visit at FLI regarding ASFV genomics sequencing collaboration (M. Sushko, SSRILDVSE). • Discussion of ASF research and control priorities in Ukraine in context of global ASF outbreaks in Eurasia and potential for cross sharing of ASF epidemiological data and ASFV genomics sequencing methods (E. Bortz, UAA). • UP9 OY1 Overview Brief distributed and electronic communication initiated.

2.6.2. Conclusion

- Methodology for library preparation and nanopore sequencing on the MinION device was developed by project researchers with guidance from

project SMEs, thereby generating ASFV and co-infection genomics data at SSRILDVSE, IVM, IECVM, NVRI in Poland, and at the University of Alaska.

- ASFV genomic sequences were analyzed and annotation was carried out for ASFV genomes in Ukraine, Poland, and the region, in support of epidemic tracing and understanding of ASF outbreaks.
- In-depth analysis of the epizootological (epidemiological) incidence and geographical data on ASF outbreaks in Ukraine (January 2019- March 2020) allowed the project team to formulate hypotheses to understand the context of ASF spread in wild boar and domestic pigs. This included mapping, outbreak clusters, trends and directions of ASF virus spread, discovering seasonality of ASF outbreaks in Ukraine, and competing explanations for spread. The importance of wild boar in persistence and re-emergence of ASF was highlighted for future study.
- Bioinformatics analysis of ASFV full genome sequences was conducted, including analysis of errors in Illumina short-read and nanopore long-read sequencing of ADFV genome to improve accuracy of reported ASFV genotypes and variants;
- Phylogenetics of multi-gene family (MGF) was performed, using comparative evolution methods for tracing the origin of the virulent ASFV/Georgia/2007 p72 genotype II lineage.
- Co-infecting pathogens in Ukraine and the region were studied, including PCV2, PEDV, PTV1, and technical models for genomic differentiation of classical swine fever and bacterial infections.
- As UP-9 has been a highly collaborative project by nature. All Ukrainian institutions (SSRILDVSE, IVM, and IECVM) have continued active collaboration and communication amongst themselves and with regional partners throughout project implementation in target working groups. These groups, mainly laboratory (sequencing), epidemiology, and bioinformatics (data analysis), supported development of research methods for analyzing ASF outbreaks in Ukraine and regional partners. Implementation required building capacity, materials, and skills for pathogen outbreak genomics.
- Continued application of genomics methods for threat reduction is warranted.
- MinION approaches provided feasible, cost-effective highly accurate pathogen genome sequencing in-country, led by Ukrainian scientists, regional partners, and project SMEs.

2.6.3. Issues or Concerns

The results obtained by the UP-9 project team compel further research. Ideally, such efforts would have been vigorously pursued during the final months of the project. However, due to the COVID-19 pandemic, a tailored approach was required, which relied on virtual communication rather than in-person meetings.

Thus, future efforts should be pursued once COVID-related restrictions are lifted to realize the full potential of this study.

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Appendix A: Funding Report

- Approved Budget: $762,883.43
- Costs to Date (30 June 2020, Direct Costs): $438,755.07
- Issues or Concerns: Final OY1 billing in underway and will be reflected in future invoicing.

UP-9 OY1 Funding Information with direct and total (direct plus indirect costs) presented.

Task Name	B&V Direct Cost	B&V Total Cost
Veterinary CBR Project UP-9 OY1 Implementation – Approved Budget	$689,892.77	$762,883.43
Veterinary CBR Project UP-9 OY1 Implementation – Cost to Date (30 June 2020)	$438,755.07	$486,315.74

Appendix B: Impact Table

UP-9 OY1: Impact Table.

Activity	Task/Team	Key Accomplishments
ASFV Genome Sequencing	1.1,1.2 / Ukrainian institutes & SMEs	• Thirteen (13) complete ASFV genomes were sequenced using MinION devices in Ukraine and one in Poland. • ASFV strains were linked in two large groups phylogenetically, which indicated two original sources of spread of ASF in Ukraine and to the region. • The first ASFV genome from Ukraine (and first ever sequenced on MinION directly from a clinical sample) was published in ASM MRA and in NCBI GenBank. • Swine co-infection viruses and bacteria were sequenced on MinION in Ukraine. • Advanced bioinformatics skills were learned and practiced by Ukrainian and regional scientists for virus genome assembly and phylogenetics.
Epidemiological Analysis of ASF Outbreaks	2.1,2.2 / Ukrainian institutes & SMEs	• Serological analysis of wild boar and domestic pig exposure to ASF was conducted. • Spatio-temporal mapping and modeling of ASF outbreaks in Ukraine revealed an important role for wild boar in spread and persistence, epidemic clustering of ASF outbreaks, transboundary threats, and seasonality.
Workshops and Scientific Advancement of Capacity	3.1,3.2 / Ukrainian institutes, NVRI, & SMEs	• Genomics and epidemiological workshops built advanced skills in pathogen genome sequencing and outbreak analysis. • Ukrainian and regional scientists, with SME, built a collaborative effort to address ASF outbreaks. • Presentations of UP-9 OY1 data in international forums were well received, including: ASM Biothreats, American Society for Virology, Nanopore Community Meeting, and DTRA SPR.

Activity	Task/Team	Key Accomplishments
Regional Collaboration	3.2 / Ukrainian institutes, regional colleagues & SMEs	• Regional scientists from Ukraine, Poland, Georgia, Armenia, and Moldova directly engaged in research in workshops in Ukraine. • Collaborative research efforts were pursued to understand transboundary threats and persistence of ASFV. • Collaborative manuscripts are in preparation.

Appendix C: UP-9 OY1 Publications and Presentations

(1) Publication at: https://doi.org/10.1128/MRA.00883-19



GENOME SEQUENCES


Complete Genome Sequence of a Virulent African Swine Fever Virus from a Domestic Pig in Ukraine

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ABSTRACT Here, we report the complete genome sequence of an African swine fever (ASF) virus (ASFV/Kyiv/2016/131) isolated from the spleen of a domestic pig in Ukraine with a lethal case of African swine fever. Using only long-read Nanopore sequences, we assembled a full-length genome of 191,911 base pairs in a single contig.

African swine fever (ASF) is a hemorrhagic viral disease of pigs that is characterized by high mortality and significant economic losses in domestic pigs. The virulent p72 genotype II lineage of African swine fever virus (ASFV; *Asfivirus*, family *Asfarviridae*) has been spreading rapidly from Georgia to Eastern and Central Europe since 2007 (1, 2). The global situation dramatically escalated, with confirmation that ASFV reached China in 2018 and had spread quickly across East Asia in 2019 (2, 3). In Ukraine, 485 laboratory-confirmed outbreaks of ASFV were recorded between 2012 and 2019 (4). The continued detection of outbreaks and the spread over the entire country raises concerns of ASFV becoming endemic in Ukraine.

Tissue samples were collected from a domestic pig from ASF outbreak number 131 in Kyiv Oblast, Ukraine, in 2016. The samples were frozen, and total DNA was extracted in duplicate from spleen tissue using the PowerMicrobiome RNA isolation kit (Mo Bio) following the manufacturer's protocol. The extraction kit retains both RNA and DNA, and a diagnostic conventional real-time PCR (RT-PCR) (LSI VetMax p72 PCR kit; Thermo Fisher Scientific) confirmed ASFV in the sample (http://www.asf.vet.ua/index.php/asfinukraine).

For full-genome sequencing, we purified the extracted DNA using Agencourt AMPure XP beads (Beckman Coulter) with three different bead-to-DNA (vol/vol) ratios (0.4×, 0.7×, and 1.0×) to retain a range of fragment lengths. We collected DNA sequence data across two sequencing runs using the Oxford Nanopore Technologies (ONT) MinION platform (Table 1). For sequencing run 1, we pooled 522 ng of DNA, including 300 ng of DNA purified from the 0.4× cleanup and 222 ng from the 0.7× cleanup. For sequencing run 2, we used 490 ng of DNA from the 1.0× cleanup. For each run, we prepared a rapid sequencing library (SQK-RAD004; ONT) and sequenced the prepared library on an R9.4.1 flow cell (FLO-MIN106) for 48 h

Citation Kovalenko G, Ducluzeau A-L, Ishchenko L, Sushko M, Sapachova M, Rudova N, Solodiankin O, Gerilovych A, Dagdag R, Redlinger M, Bezymennyi M, Frant M, Lange CE, Dubchak I, Mezhenskyi AA, Nychyk S, Bortz E, Drown DM. 2019. Complete genome sequence of a virulent African swine fever virus from a domestic pig in Ukraine. Microbiol Resour Announc 8:e00883-19. https://doi.org/10.1128/MRA.00883-19.

Editor Jelle Matthijnssens, KU Leuven

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Received 13 August 2019
Accepted 17 September 2019
Published 17 October 2019

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TABLE 1 Summary of sequencing data statistics

Data set	No. of reads	Yield (bp)	Avg length (bp)	Avg quality (Q score)
Run 1 raw	2,213,244	5,213,092,675	2,355.4	10.5
Run 1 quality controlled	1,649,561	3,994,767,329	2,421.7	13.1
Run 2 raw	3,290,571	6,235,197,650	1,894.9	10.5
Run 2 quality controlled	2,430,267	4,631,455,885	1,905.7	13.1
Total quality controlled	4,079,828	8,626,223,214	2,114.4	13.1
After *S. scrofa* removed	98,078	27,426,602	279.6	12.5

using a MinION Mk1B device. We base called raw data using Guppy v3.1.5 (ONT) with a high-accuracy model (dna_r9.4.1_450bps_hac.cfg) and default parameters. Before assembly, we created a quality-controlled data set using Porechop v0.2.4 (https://github.com/rrwick/Porechop) with default parameters to trim adaptors and discard sequences with middle adapters (-discard_middle) and Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) to filter by a quality (Q) score of ≥10 (-min_mean_q 90).

To remove reads likely originating from the host, we used Minimap2 v2.17-r941 (4) with default parameters for Nanopore reads (-ax map-ont) to align our quality-controlled data to the *Sus scrofa* 11 reference genome (GenBank assembly accession no. GCA_000003025). We extracted the unmapped reads for *de novo* assembly using SAMtools v1.9 (5). We assembled the genome using Flye v2.4.2 (6) with default parameters specifying the estimated genome size (-genome-size = 200k) and Nanopore reads (-nano-raw). Our raw assembly (coverage, 32×) consists of 227,741 bp in 9 linear contigs (N_{50}, 193,207 bp). We confirmed via an NCBI blastn search (7) that only the longest contig, 193,207 bp, was ASFV. The top hits for this contig had a greater than 99% identity to recently published ASFV genomes (e.g., GenBank accession no. LR536725 and MK128995). For this longest contig, we used a polishing pipeline specific to the error profile of Nanopore reads. We completed two rounds of polishing with the graphics processing unit (GPU)-enabled version of Racon v1.3.3 (https://github.com/clara-genomics/racon-gpu) (8) with the following parameters: score for matching bases (-match 8), score for mismatching bases (-mismatch -6), threshold for average base quality of windows (-quality-threshold -1), default gap penalty (-gap -8), default window (-window-length 500), and number of Compute Unified Device Architecture (CUDA) batches (-c 2). We completed a final round of Nanopore-specific polishing with Medaka v0.8.0 (https://github.com/nanoporetech/medaka).

Our polished assembly (coverage, 27×) consists of 191,911 bp in a single linear contig (GC content, 38.3%). We confirmed that this is a Georgia lineage p72 genotype II strain by using blastn (7) to compare the sequence variation at the p72 (B646L) gene and found 99.9% identity to Georgia 2007/1 (GenBank accession no. FR682468), with the only difference associated with two different homopolymer regions. We used MAFFT v7.388 (9, 10) to align our polished assembly with the Georgia 2007/1 assembly. A visual inspection of this alignment with Geneious Prime v 2019.1.1 confirmed overlap of the complete genome, including ends. We found a 10-nucleotide insertion (GGAA TATATA) between the I73R and the I329L genes, as previously reported (11–13). According to those previous reports, this insertion is not linked to attenuation or virulence. This insertion is present in the China/2018/AnhuiXCGQ genome (GenBank accession no. MK128995) but absent from ASFV/POL/2015/Podlaskie (accession no. MH681419) and Georgia 2007/1. We did not detect the left-end genome deletion found in an attenuated strain from Estonia (14). This is the first full-genome report of a virulent Georgia lineage p72 genotype II strain from the multiyear zoonotic outbreaks of ASF in Ukraine, highlighting the accuracy and deployability of Nanopore sequencing on a MinION platform for analysis of an emerging disease that has spread across Eurasia.

Data availability. This genome sequence has been deposited in GenBank under the accession no. MN194591. The version described in this paper is the first version, MN194591.1. Raw data for this project can be found in the GenBank SRA under accession no. PRJNA555080.

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ACKNOWLEDGMENTS

Many thanks to Natalia Usachenko, Maryna Kit, Xiao Bai, and Jeremy Buttler for technical support; Grzegorz Woźniakowski and Jürgen Richt for helpful discussions; and to Natalia Gudz, Nataliya Mykhaylovska, Olga Fedorenko, Karen Hite, David Mustra, and Mary Guttieri for project support.

This work was funded by the U.S. Defense Threat Reduction Agency (DTRA) through the Biological Threat Reduction Program in Ukraine (Ukraine Project-9). The contents of this publication are the responsibility of the author and do not necessarily reflect the views of DTRA or the United States Government. Research reported in this publication was in part supported by an NIH NIGMS Institutional Development Award (IDeA), grant no. P20GM103395 (Alaska INBRE). We also acknowledge the generous support of the Institute of Arctic Biology at UAF and that of the UAA College of Arts and Sciences.

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(2) Poster presentations at the 2019 ASM Biothreats, 29-31 January 2019, Arlington, USA

- **Abstract for Poster 1**

Spatial Analysis of the Spread of African swine fever in Ukraine

M. Bezymennyi [1], C. Lange [2], A. Kovalenko [1], M. Sushko [3], N. Hudz[1], M. Sytiuk[1], M. Sapacheva [3], A. Mezhensky [3], E. Bortz [4], S. Nychyk [1].

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The spread of African swine fever (ASF) in Ukraine brings heavy losses to the pig farming which can negatively affect the food security of the country. Since 2012, there have been 431 registered outbreaks of the disease (as of 21 Sep 2018). The extent and ways of spreading the virus in Ukraine are not clear enough. In this study, we conducted spatial analysis of ASF outbreaks in wild boars and domestic swine using descriptive spatial statistics in GIS: ellipses of standard deviations, kernel density estimation. To assess the possible human factor in the spread of the disease, we analyzed the distances between outbreaks. We also analyzed the distances taking into account the time it took for the virus to travel that distance. The first case of ASF in Ukraine was recorded in 2012 in the backyard in Zaporizhzhia Oblast in the South-East of the country. At that time, the Ukrainian veterinary service managed to localize the disease and prevent its further dissemination. The next outbreaks appeared in 2014 in the East and North-East of Ukraine not far from the border with the Russian Federation. In 2015, the infection moved from the North-East to the center of the country and reached all regions of Ukraine in subsequent years. In 2018 (as of the end of September), the largest ASF clusters are located in the South, South-West and West of Ukraine in the border areas with Moldova, Romania, Hungary, Slovakia and Poland. The highest density of ASF outbreaks among wild boars is in the West and South-West of the country. The distance analysis carried out on official data showed that in some cases the virus "jumped" over a distance of about 295 km from previous outbreaks. In such cases, we can more confidently assume the impact of human activity on the spread of ASF virus.

- **Abstract for Poster 2**

Genomic sequencing of virulent African swine fever virus in Ukraine

M. Sapacheva [1], M. Sushko [1], N. Usachenko [1], G. Kovalenko [2], L. Muzykina [2], M. Bezymenyi [2], O. Solodiankin [3], N. Rudova [3], O. Pishchanskyi [1], A. Mezhenskyi [1], C. Lange [4], J. Buttler [5], R. Dagdag [5], A. Ducluzeau [6], D. Drown [6], E. Bortz [5].

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African swine fever (ASF) is a highly contagious hemorrhagic disease that causes high mortality in pigs and results in significant losses for the pork industry. Within the state surveillance activity of Ukraine, 4,944 samples from wild boar and 21,381 from domestic animals were studied. Using PCR, 163 suspected cases were confirmed positive for ASF virus (ASFV) in 2017 and 123 in 2018.

Using nanopore sequencing and bioinformatics, in-depth study of ASFV in Ukraine was initiated under research project UP-9 supported by the US Defense Threat Reduction Agency's Cooperative Biological Engagement Program. The efficacy of two nanopore sequencing library preparation protocols tailored for PCR amplicons and whole genome sequencing were tested. Using an ASFV-positive sample, 13 loci, representing 22 genes in one novel strain isolated from domestic pigs in Ukraine (ASFV/Zakarpattia/243/2017), were sequenced on the MinION using the LSK-108 library preparation. Amplicon sequencing and whole genome sequencing using the rapid library preparation were also completed on another strain, ASFV/Chernihiv/2014/7. Despite the high abundance of reads matching the host, 30 times coverage of the entire ASFV genome was recovered with only eight hours of sequencing. These two strains were closely related to the virulent ASFV/Georgia/2007, p72-genotype II lineage, providing a genomic look into the ASF outbreaks in Ukraine.

The development of protocols for analysis of genomic signature sites in the ASFV genome, as well as improvement of reporting among the project participants and collaborators, will provide relevant information for ASF outbreak control.

(3) UP-9 Oral Presentation at the 38th Annual ASV Meeting, 20-24 July 2019, MN, USA

- **Abstract**

Conserved genome architecture of virulent African swine fever virus (ASFV) in Ukraine from long read nanopore sequencing.

Eric Bortz[1,2], Mykola Sushko[3], Anna Kovalenko[2], Anne Lise Ducluzeau[4], Matthew R. Redlinger[1], Xiao Bai[1], Ralf Dagdag[1], Maciej Frant[6], Christian E. Lange[5], Devin M. Drown[4], Maryna Sapacheva[3], Andrii A. Mezhenskyi[3]
[1]University of Alaska Anchorage; [2]Institute for Veterinary Medicine, Kyiv Ukraine; [3]State Institute for Veterinary Laboratory Diagnostics (SSRILDVSE), Kyiv Ukraine; [4]University of Alaska Fairbanks; [5]Metabiota, Inc.; [6]National Veterinary Research Institute, Pulawy Poland

African swine fever virus (Asfarviridae) infects monocytes and macrophages, causing a lethal hemorrhagic infection in domestic pigs and wild boar. Virulent ASFV (p72-genotype II) emerged in Georgia in 2007, with 189kbp dsDNA genome and 150+ genes. Since 2014, Ukraine has suffered almost 500 outbreaks of ASF. However, the ability to trace ASF epidemics is limited by incomplete understanding of ASFV genetics and pathogenesis.

To better understand ASFV genetics, we sequenced full genomes of 7 ASFV isolates from across Ukraine using long read nanopore sequencing technology. Total DNA was purified from spleen of animals suffering from clinical ASF. A rapid end-tagged DNA library, and barcoded PCR amplicon libraries, were sequenced using an ONT MinION device. Following basecalling, complete genomes of 7 ASFV isolates were recovered using reference-guided sequence assembly. Two geographically and temporally distant strains were analyzed to understand genotypic variation: domestic pig ASFV/Chernihiv/2014/7 and wild boar ASFV/Odessa/2018/398. Remarkably, these isolates harbored only 5 and 8 genotypic variations from the ASFV/Georgia/2007 reference strain, respectively. Three SNP appeared in common: an 18 amino acid C-terminal truncation in MGF 110-1L removed putative heparin sulfate binding but retained a RINGv domain; N414S in the NP419L DNA ligase OB-fold, and K323E in MGF 505-9R, a putative interferon evasion protein, were also found in ASFV isolated in Estonia and Poland. Unlike other ASFV/Georgia/2007 isolates containing left-end deletions, both Ukraine isolates contained the original set of multigene family (MGF 110, 360 and 505) genes. Phylogenetics analyses of MGF 110 and MGF 360 genes in ASFV genotypes I, II, IX, and X suggest a conserved architecture and discrete gene duplication events. Terminal repeat-spanning reads suggest genome circularization. Taken together, the conservation and genome architecture of ASFV suggests a DNA replication cycle that preserves viral pathogenicity and transmissibility but restricts host range in a broadening outbreak.

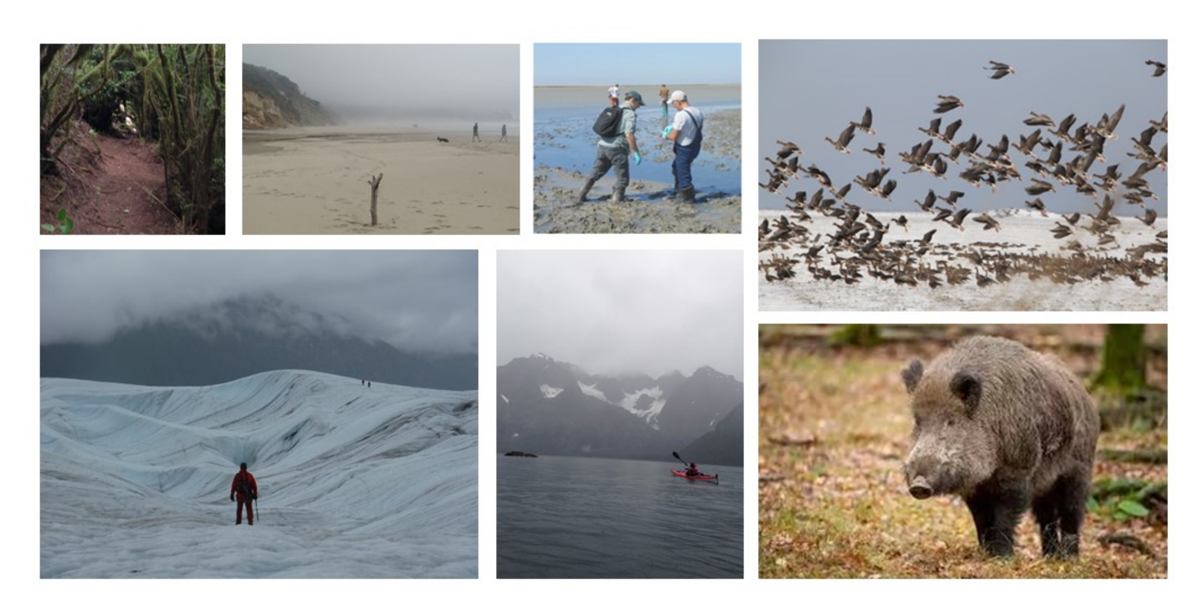
- **Oral Presentation**

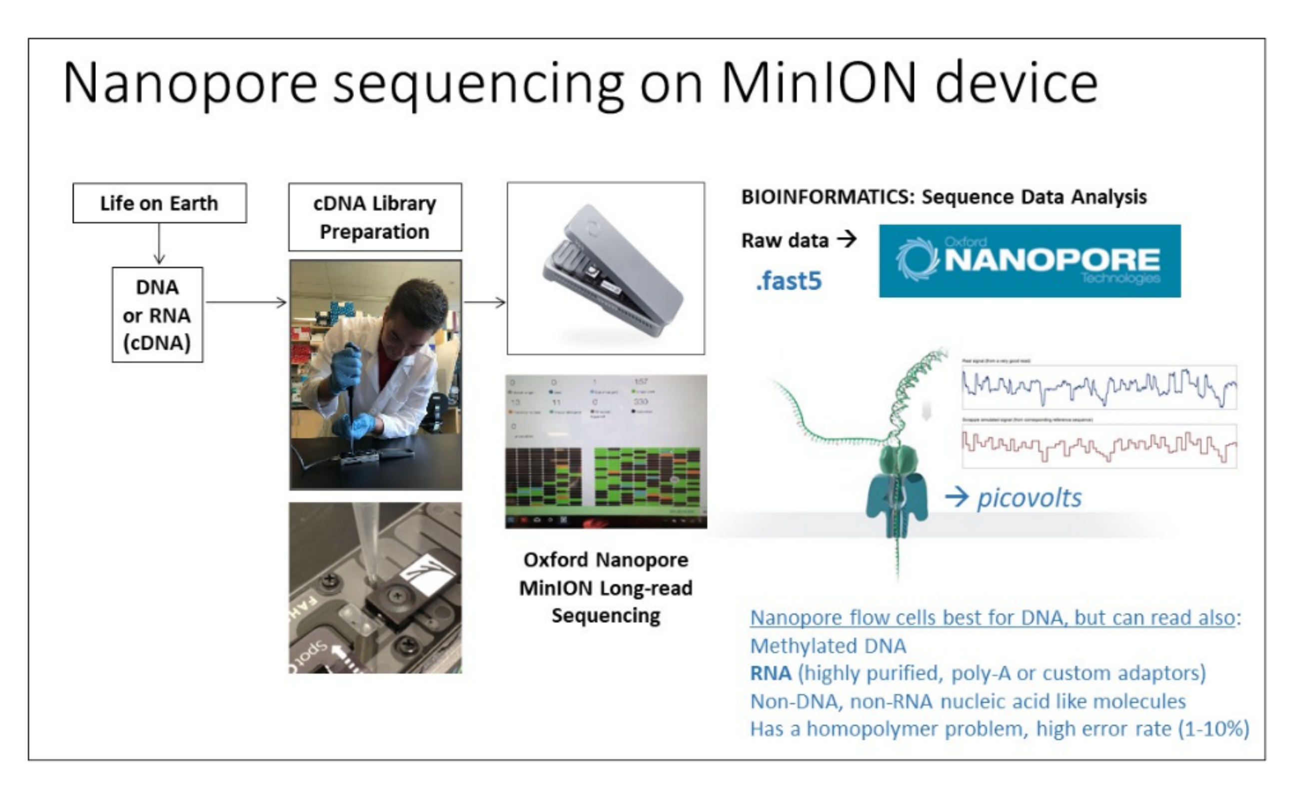


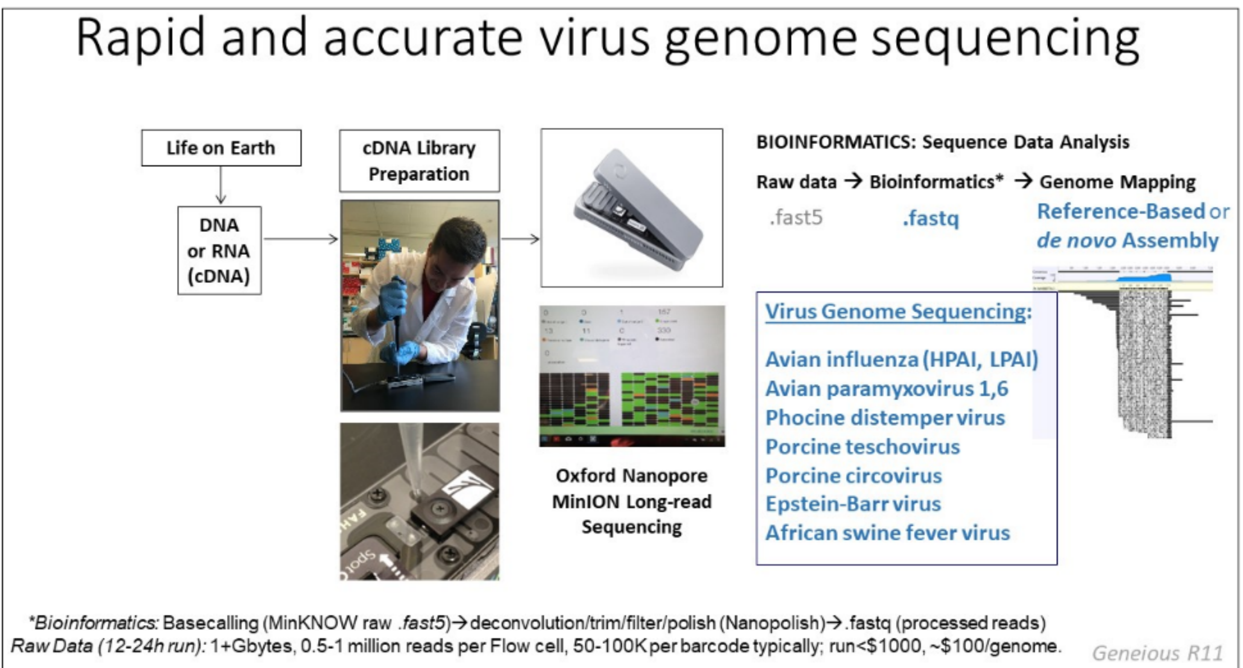
Conserved genome architecture of virulent African swine fever virus (ASFV) in Ukraine from long read nanopore sequencing.

Eric Bortz, Ph.D.
Assistant Professor
Department of Biological Sciences
University of Alaska Anchorage
ebortz@alaska.edu
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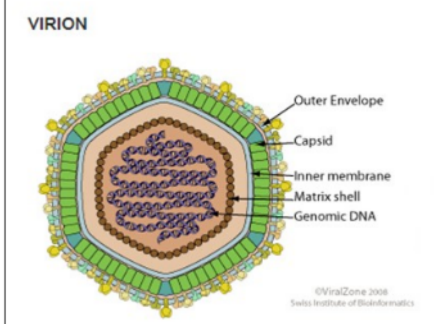
Viruses are the dark matter of the biosphere




Nanopore sequencing on MinION device
Life on Earth
DNA or RNA (cDNA)
cDNA Library Preparation
Oxford Nanopore MinION Long-read Sequencing
BIOINFORMATICS: Sequence Data Analysis
Raw data →
.fast5
Oxford NANOPORE Technologies
→ picovolts
Nanopore flow cells best for DNA, but can read also:
Methylated DNA
RNA (highly purified, poly-A or custom adaptors)
Non-DNA, non-RNA nucleic acid like molecules
Has a homopolymer problem, high error rate (1-10%)

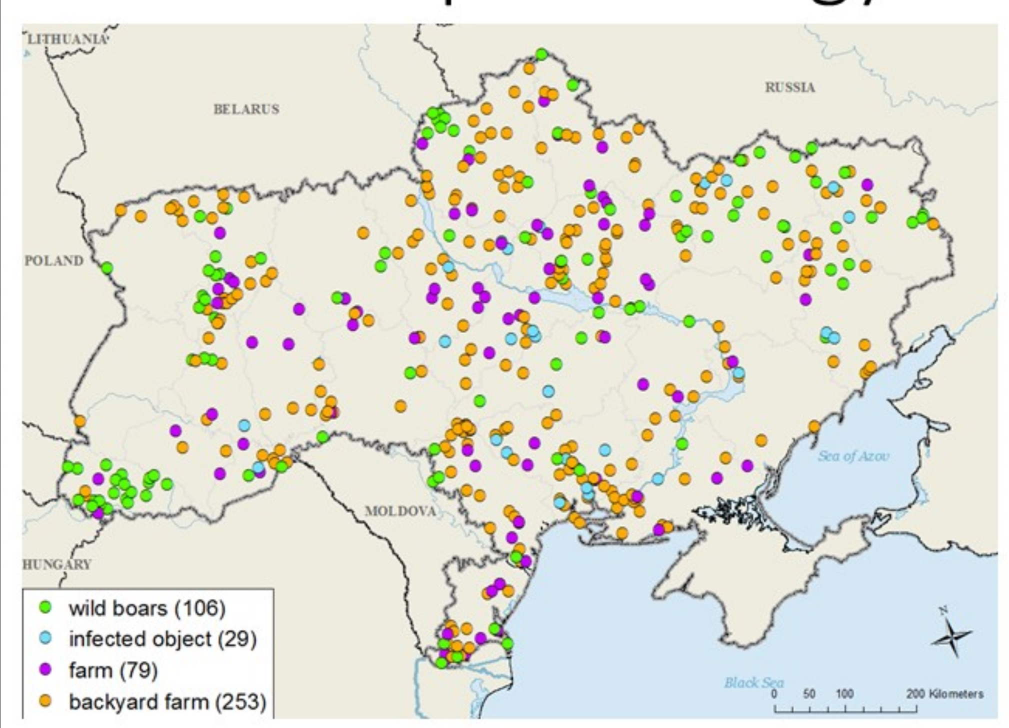

Rapid and accurate virus genome sequencing
Life on Earth
DNA or RNA (cDNA)
cDNA Library Preparation
Oxford Nanopore MinION Long-read Sequencing
BIOINFORMATICS: Sequence Data Analysis
Raw data → Bioinformatics* → Genome Mapping
.fast5
.fastq
Reference-Based or de novo Assembly
Virus Genome Sequencing:
Avian influenza (HPAI, LPAI)
Avian paramyxovirus 1,6
Phocine distemper virus
Porcine teschovirus
Porcine circovirus
Epstein-Barr virus
African swine fever virus
*Bioinformatics: Basecalling (MinKNOW raw .fast5)→deconvolution/trim/filter/polish (Nanopolish)→.fastq (processed reads)
Raw Data (12-24h run): 1+Gbytes, 0.5-1 million reads per Flow cell, 50-100K per barcode typically; run<$1000, ~$100/genome.
Geneious R11

Virulent African swine fever virus (ASFV) outbreaks in Eurasia



- **African swine fever (ASF): lethal hemorrhagic disease in Suids (pigs).**
- Large dsDNA virus (170-190kbp) with >150 genes.
- 24 ASFV genotypes.
- Virulent ASFV/Georgia-lineage (p72-genotype II) spread 2007-2019 from the Caucasus to Russia, Ukraine, Poland, Belgium, China & southeast Asia
- *Vector*: Argasid ticks (*Ornithodoros* species). The virus can replicate within the ticks (but not found in European ASF).
- *Cell tropism:* macrophages and monocytes.
- Immunomodulatory genes (interferon antagonism).
- There is as yet no effective vaccine.
- There are no effective licensed antiviral drugs.

Tracking ASF outbreaks: molecular epidemiology



ASF outbreaks in Ukraine (2012-2019)

Source: Govt. of Ukraine SSRILDVSE lab: http://www.asf.vet.ua/index.php/asfinukraine

Tracking ASF outbreaks: molecular epidemiology

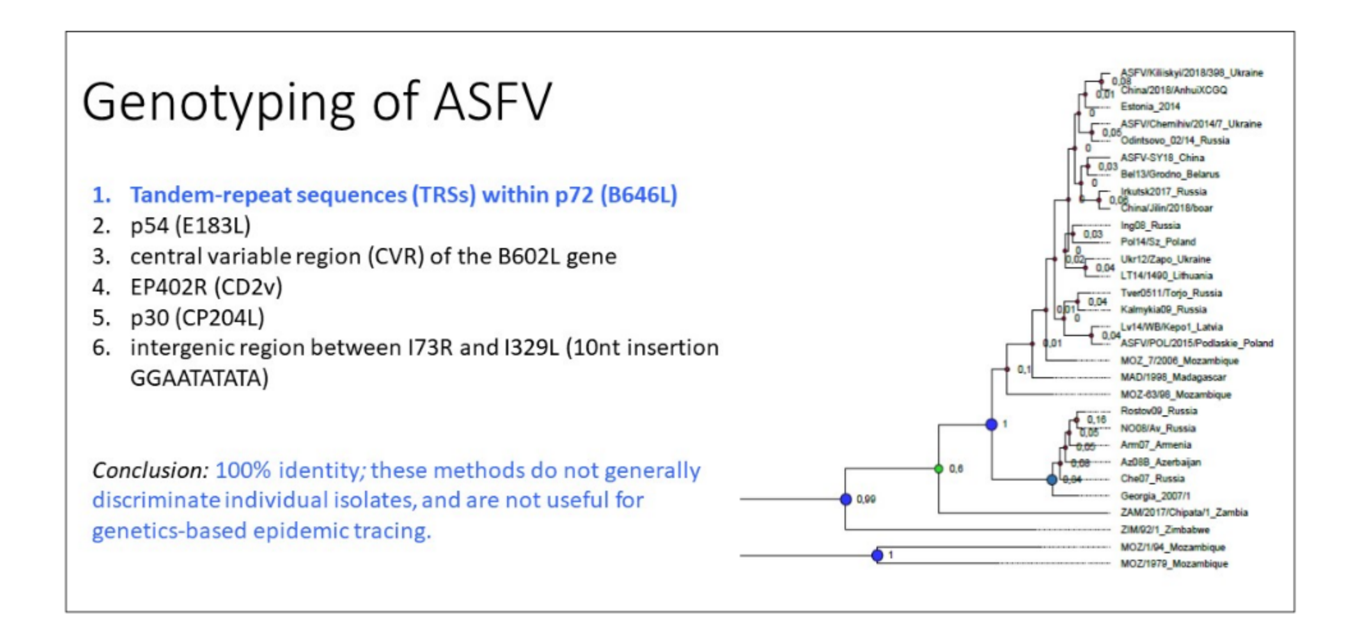
Wild Boar (2014-18)

A

Domestic Pigs (2014-18)

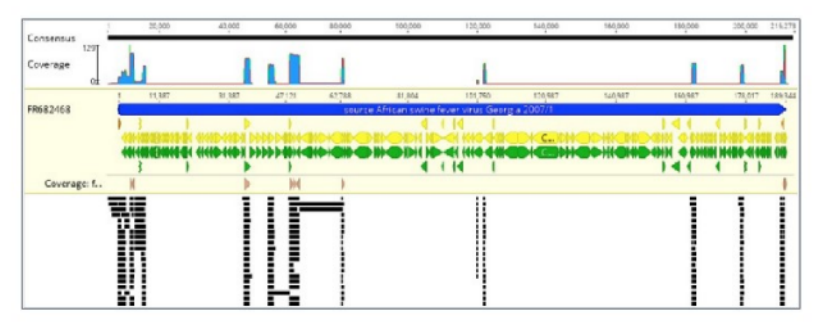
Value
High : 0.000467384
Low : 0

B



Sequencing genomic signatures (SNP) of ASFV in Ukraine

Sequence a series of amplicons across discriminatory loci in the the genome ("genomics signatures") … barcode, multiplex and nanopore sequence many outbreak samples simultaneously.

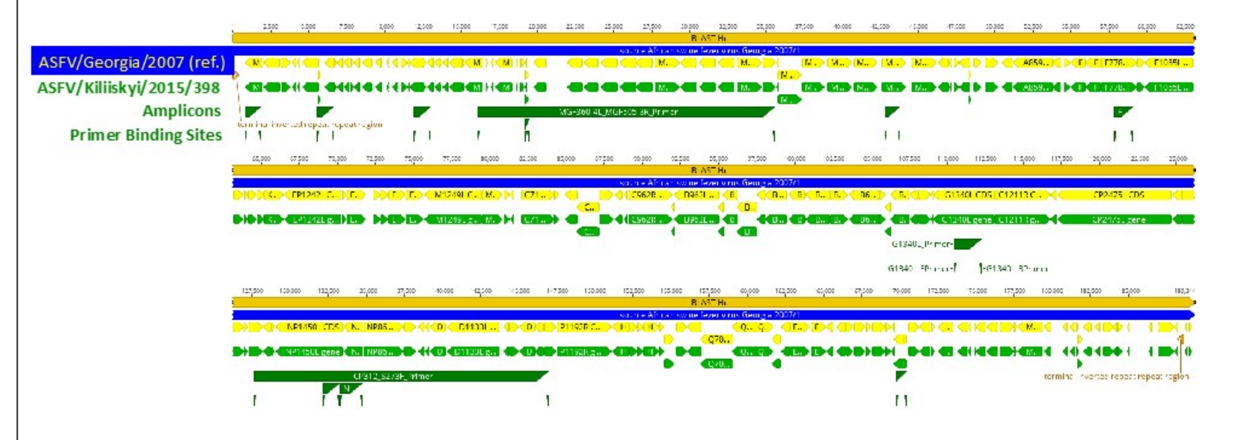


Amplicon sequencing of ASFV/Zakarpattia/2017/243

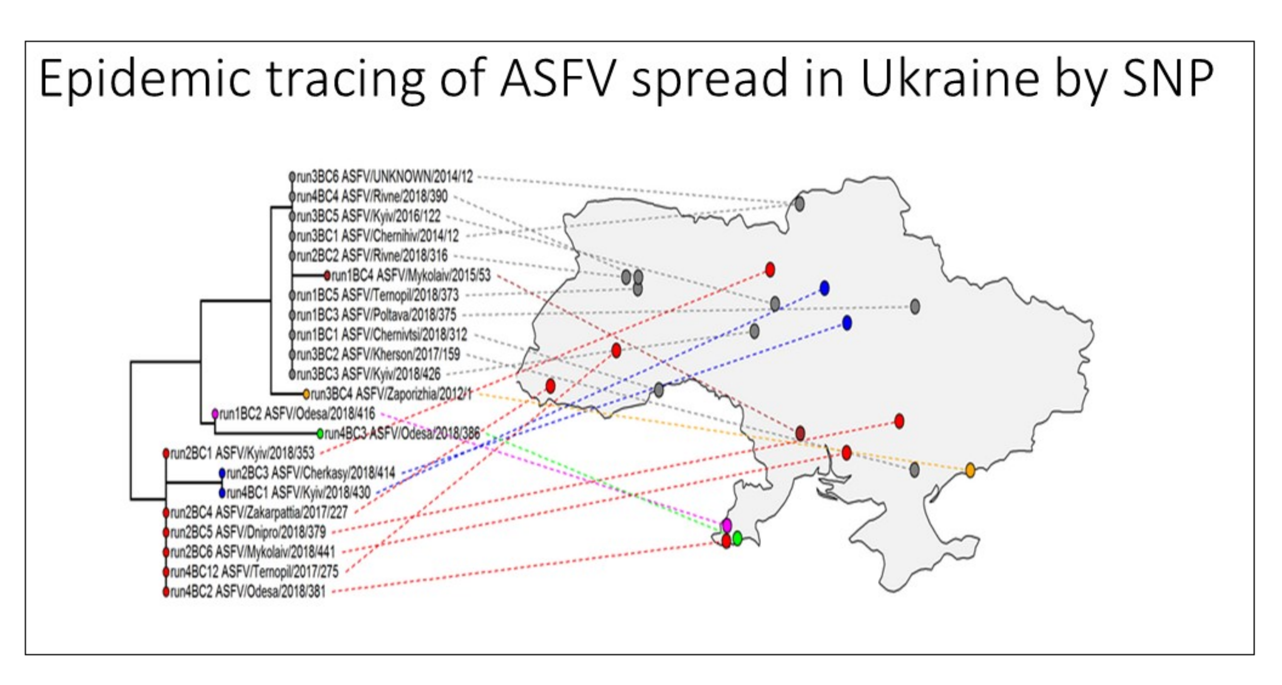
Identification: **virulent ASFV/Georgia/2007-lineage, p72-genotype II**

Epidemic tracing of ASFV spread in Ukraine by SNP

Design SNP flanking primers→ PCR clinical samples → MinION



ASFV does not mutate a lot: 6-8 SNP per isolate.



What about full genome sequencing of ASFV?

RNA virus: Newcastle disease virus (La Sota/vaccine preparation)

Consensus
Coverage
AF077761.1

DNA virus: Epstein-Barr virus (latent genome/Raji cells)

Consensus
Coverage

So far 7 full ASFV genomes sequenced from clinical samples (spleen from animals found dead of ASF)

Bioinformatics pipeline

Basecalling → Guppy → Raw Reads

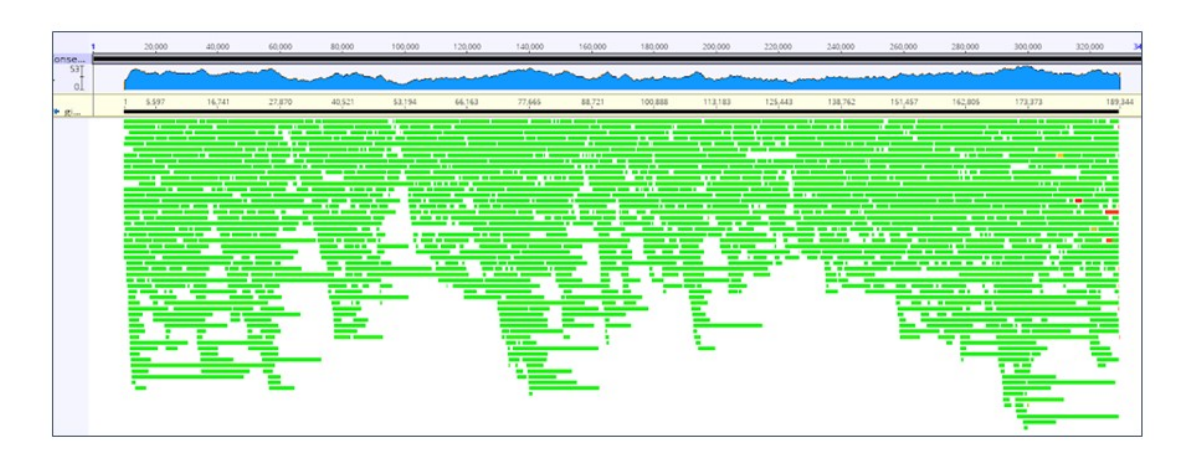
Quality control → Porechop → Filtlong → QC Reads

Alignment to Virus Genomes → • Georgia • Poland • China → Variant calling → Reference based Genome

Alignment to Host Genome → Unmapped reads → de novo assembly → Genome polishing → de novo polished Genome

Variant Calling: identification of SNP, indels, and genome variations

So far 7 full ASFV genomes sequenced from clinical samples (spleen from animals found dead of ASF)



189kbp dsDNA genome of ASFV/Chernihiv/2014/7 (30X)

ASFV sequenced from both domestic pig and wild boar: ASFV/Chernihiv/2014/7 and ASFV/Kiliiskyi/2018/356

	Chernihiv/2014	Kiliiskyi/2018
Host	Domestic Pig	Wild Boar
Raw Yield	2 Gbases	0.9 Gbases
Duration	8 hours	24 hours
QC Yield	514 Mbases	600 Mbases
Mapped	862 reads	1724 reads
Coverage	24x	60x

Annotating the full genome of ASFV in Ukraine

1 20,000 40,000 60,000 80,000 100,000 120,000 140,000 160,000 189,344

source African swine fever virus Georgia 2007/1

C

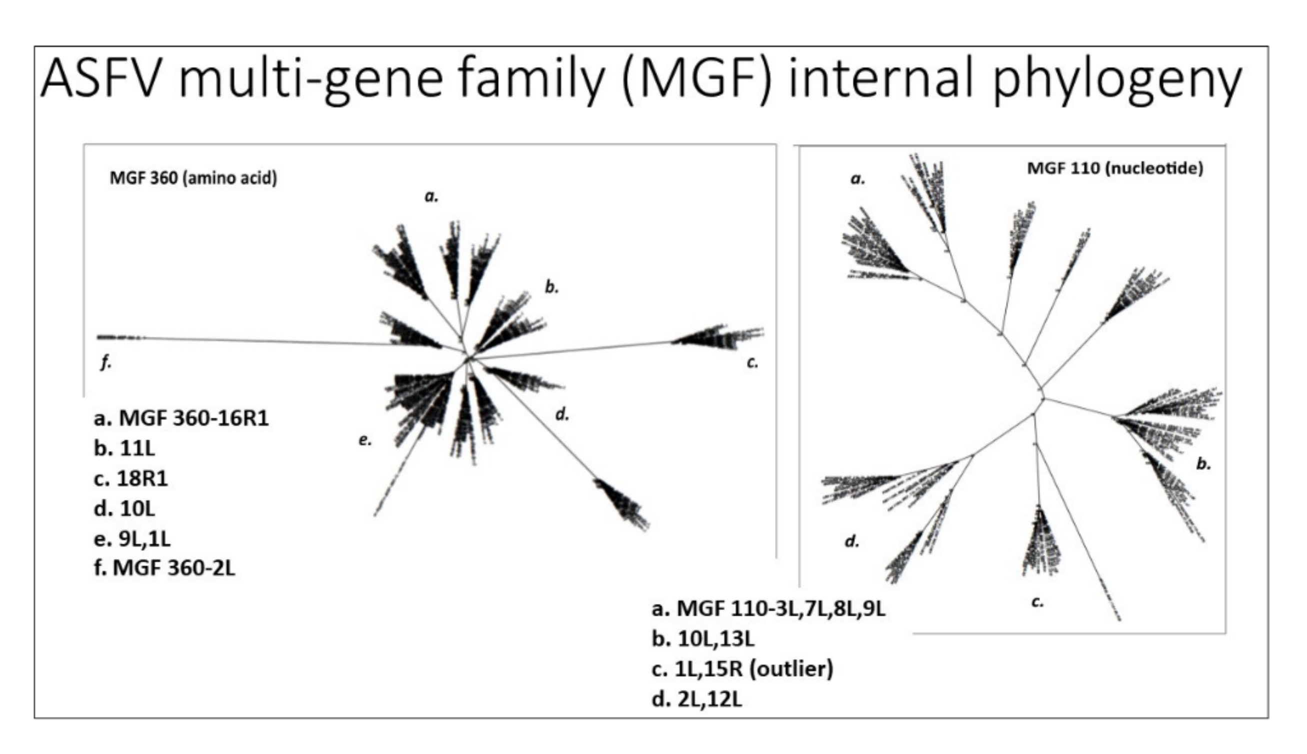
C

terminal inverted repeat repeat region

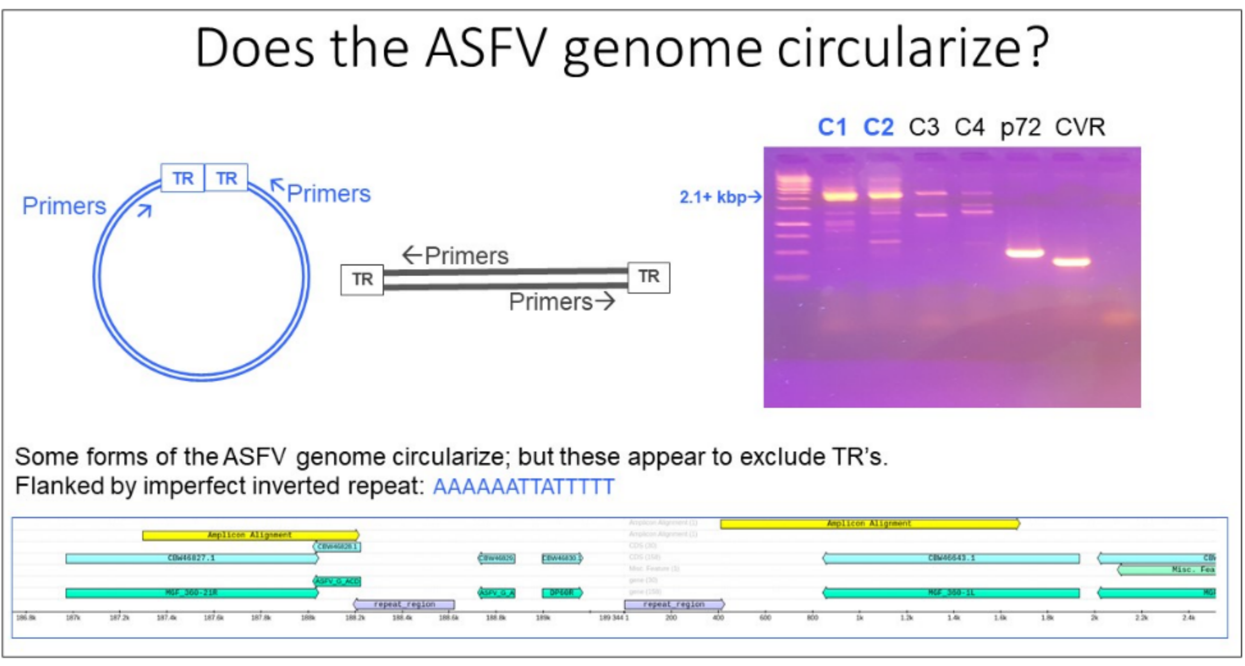
terminal inverted repeat repeat region

Genome Annotation: 189kbp dsDNA genome of ASFV/Chernihiv/2014/7

MGF 505-9R (putative interferon antagonist): K323E

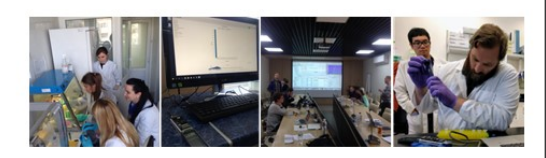


ASFV multi-gene family (MGF) internal phylogeny
MGF 360 (amino acid)
a. MGF 360-16R1
b. 11L
c. 18R1
d. 10L
e. 9L,1L
f. MGF 360-2L
MGF 110 (nucleotide)
a. MGF 110-3L,7L,8L,9L
b. 10L,13L
c. 1L,15R (outlier)
d. 2L,12L



Does the ASFV genome circularize?
Primers
TR
TR
Primers
←Primers
TR
TR
Primers→
C1 C2 C3 C4 p72 CVR
2.1+ kbp→
Some forms of the ASFV genome circularize; but these appear to exclude TR's.
Flanked by imperfect inverted repeat: AAAAAATTATTTTT
Amplicon Alignment
Amplicon Alignment
repeat_region
repeat_region

Summary.



- We have been sequencing virulent African swine fever viruses from clinical samples in Ukraine using nanopore (MinION) technology.
- ASFV does not mutate alot, but enough for epidemic tracing using SNP. Traditional ASFV genotyping markers are insufficient for this.
- ASFV in Ukraine has spread in multiple waves from what appear to be growing centers of endemicity; in wild boar and domestic pigs.
- Roles of genome variations in pathogenicity and transmission: *unknown*.
- ASFV full genome (189kbp) can be sequenced directly from clinical samples.
- The ASFV genome can circularize *in vivo*.
- It takes a village. Multiple kinds of expertise in the team.
- Thanks!

Achnowledgements.



UAA: Eric Bortz Lab
Matthew Redlinger
Amy Klink
Ralf Dagdag
Xiao Bai
Jeremy Buttler
Cora Lyon
Brandon Maniaci
Will George

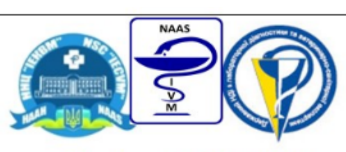
Holly Martinson (EBV)



UAF: Devin Drown Lab
Anne Lise Ducluzeau

NVRI Poland: Greg Wozniakowski, Maciej Frant

Christian Lange (Metabiota)
Inna Dubchak (LBNL)



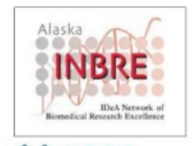
Ukraine: SSRILDVSE
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Maryna Sapachova
Natalia Usachenko
Andij Mezhenskiiy

Ukraine: IVM
Ganna Kovalenko
Maksym Bezyemenni
Lorysa Muzykina

Ukraine: IECVM
Anton Gerilovych
Nataliia Rudova
Maryna Kit
Oleksii Solodiankin

Funding Support









METABIOTA

Research reported in this presentation was in part supported by the DTRA Cooperative Biological Engagement Program (CBEP); an Institutional Development Award (IDeA) from NIGMS (P20GM103395); and NIAID Centers of Excellence in Influenza Research and Surveillance (CEIRS) award (HHSN272201400008C). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH or the US Dept. of Defense.

(4) UP-9 Presentations at the DTRA Science Program Review, 19-20 September 2019, Poland

- **UP-9 Oral Presentation**

The Spread of African Swine Fever Virus (ASFV) in Domestic Pigs and Wild Boar in Ukraine – Building Capacity for Insight into the Transmission of ASFV through Characterization of Virus Isolates by Genome Sequencing and Phylogenetic Analysis

Presenter: **Dr. Anna Kovalenko**

Institute of Veterinary Medicine of the National Academy of Agrarian Sciences
Kyiv, Ukraine

Project Participants and Collaborators

Project Participants in Ukraine

- State Scientific Research Institute of Laboratory Diagnostics and Veterinary Sanitary Expertise (SSRILDVSE), Kyiv
- Institute of Veterinary Medicine (IVM) of the National Academy of Agrarian Sciences (NAAS) of Ukraine, Kyiv
- National Scientific Center Institute of Experimental and Clinical Veterinary Medicine (IECVM), Kharkiv

International Collaborators

- University of Alaska Anchorage (UAA), Anchorage, AK, USA
- University of Alaska Fairbanks (UAF), Fairbanks, AK, USA
- U.S. Department of Energy Joint Genome Institute (JGI), Lawrence Berkeley National Laboratory (LBNL), USA
- Metabiota, Inc. (Metabiota), San Francisco, CA, USA
- National Veterinary Research Institute (NVRI), Puławy, Poland















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Technical Description and Background

Technical Description

Project focus: Advancing ASFV genome sequencing capabilities, genome-based biosurveillance analysis in Ukraine, and enhancing reginal collaborative network.

Hypotheses:

(1) ASF outbreak dynamics in swine species can be epidemiologically traced by nanopore sequencing of ASFV isolates to generate genomic signatures of emerging ASFV strains;

(2) Characterization of Ukrainian ASFV genomes will identify sequence variations for the analysis of prevalence and regional spread of virus within wild boar and domestic pigs in Ukraine and countries in the region

Current Understanding

Base period of project performance has led to the following corollary hypothesis: Emergent ASFV genotype variants in Ukraine may have different pathogenicity, transmission patterns, and/or longer persistence in swine populations, increasing risk of virus spread and persistence in the susceptible population

3

Europe
Wild boar in Poland, Baltics & Belgium
485 swine outbreaks in Ukraine
Romania
Current ASF outbreaks: Eurasia
Asia
22% (60+ million) pigs culled in China
2 million culled in Viet Nam

Virulent ASFV/Georgia-lineage (p72-genotype II) spread 2007-2019

OIE Report #21 ASF July 2019

Threat Reduction Objectives

Threat Reduction Objectives

- Deploy state-of-the-art genomic-based biosurveillance for African swine fever (ASF) to better understand of risk factors
- Enhance the capacity for ASF detection, response, and control in Ukraine
- Identification of internal and cross-border origins of virulent ASF virus (ASFV) in order to mitigate the threat of ASF as a severe disease of multi-national concern
- Improve technical capacity for computational (bioinformatics) analysis of ASFV sequences for epidemiological mapping and robust, accurate ASFV strain identification to anticipate and respond to the spread of ASF in Ukraine and across borders in the region
- Integration of Ukrainian scientists and Institutes into the international scientific community by helping to strengthen the country's especially dangerous pathogen detection and response networks and by adoption of international diagnostic standards, publication and dissemination of results



Key Partners and Regions of Study

Key Partners

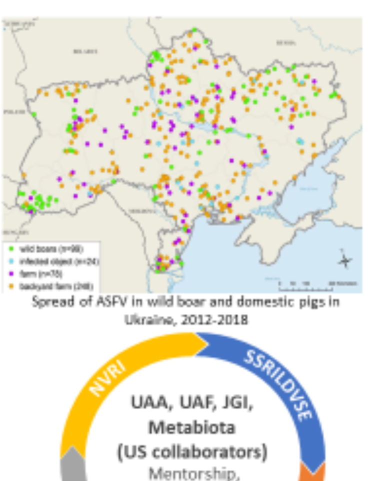
- General Coordination, Mentorship, Workshops, Training, Data Analysis and Integration – **UAA, UAF, JGI, Metabiota**
- Building Regional Partnerships, Serological Surveillance – **NVRI**
- Coordination, Sample Collection, ASF PCR Diagnostics, Serological Surveillance, Genome Base Biosurveillance – **SSRILDVSE**
- PCR/RT-PCR/sequencing, Genome analysis, Study of Co-infections – **IVM, IECVM, SSRILDVSE, UAA, UAF**
- GIS Mapping and Modeling, Data Server – **IVM, UAF, Metabiota**

Regions

Samples are collected through the existing veterinary infrastructure of State Service of Ukraine on Food Safety and Consumer Protection. Samples are selected from ASF outbreaks in Ukraine (2012-2019)

Networks

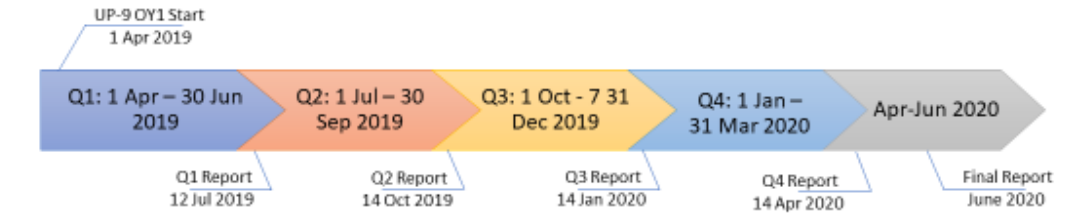
- Ukrainian Collaborators: SSRILDVSE, IECVM, IVM
- US Collaborators: UAA, UAF, JGI, Metabiota
- Regional Collaborators: NVRI



Spread of ASFV in wild boar and domestic pigs in Ukraine, 2012-2018

5/15

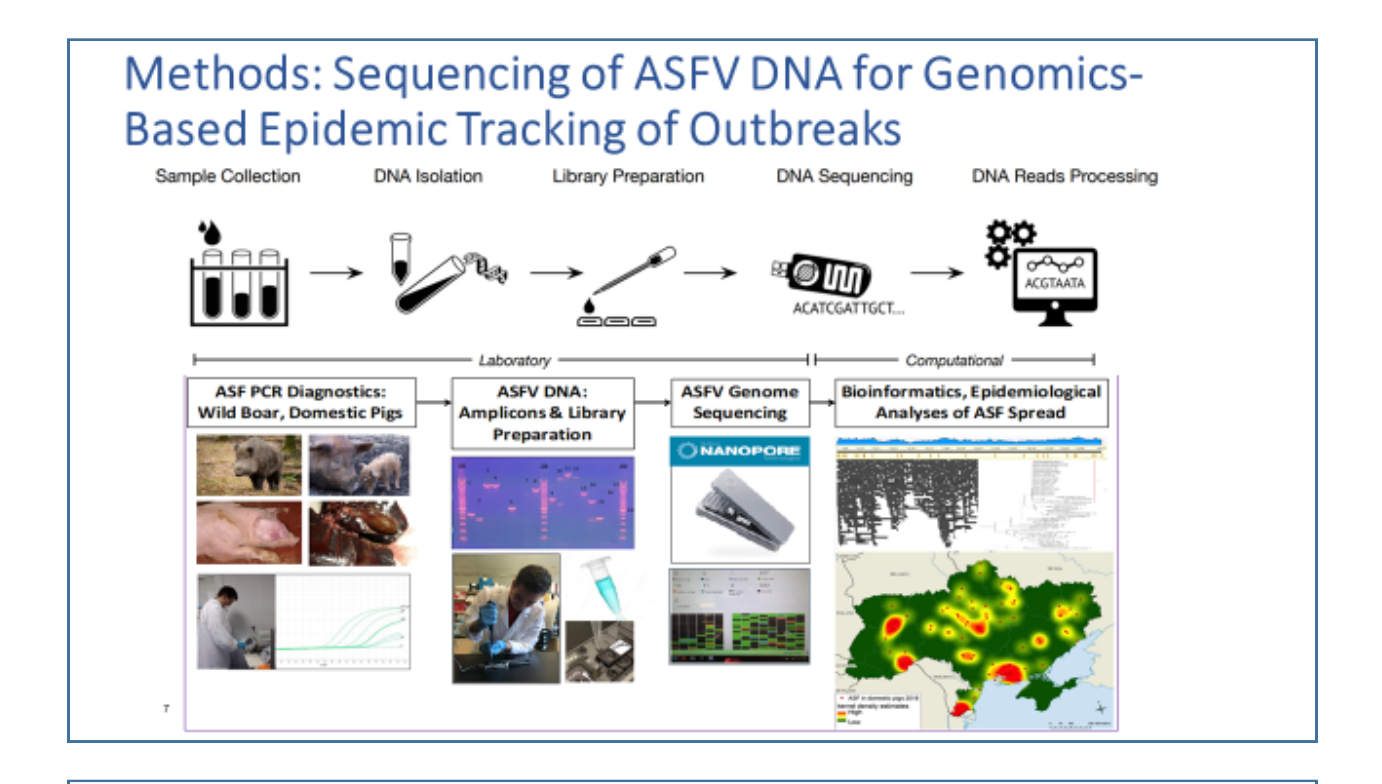
Timeline and Major Milestones



- Study of ASFV genotype variation: April 2019 – March 2020
- Full genome sequencing using MinION: April 2019 – March 2020
- ASF outbreak mapping: April 2019 – March 2020
- Bioinformatics: April 2019 – March 2020
- Scientific Advancement Workshops: May 2019, June 2019, September 2019, December-January 2020 (TBD), March 2020 (TBD)

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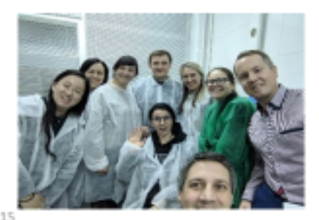


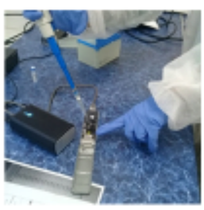


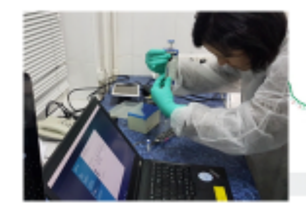
ASFV Genome Sequencing Results

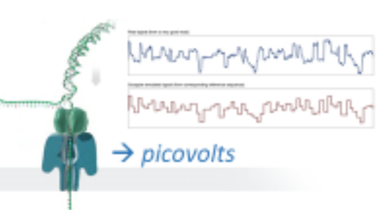
Key Accomplishments for the base period:

- Generated first deployable genotyping approach for ASFV in Ukraine
- Three full ASFV genomes were sequenced
- Nine partial ASFV genomic signatures using long-read nanopore technology
- Assembled complete genomes of 2 ASFV strains from Ukraine:
- Initiated development of 2 manuscripts









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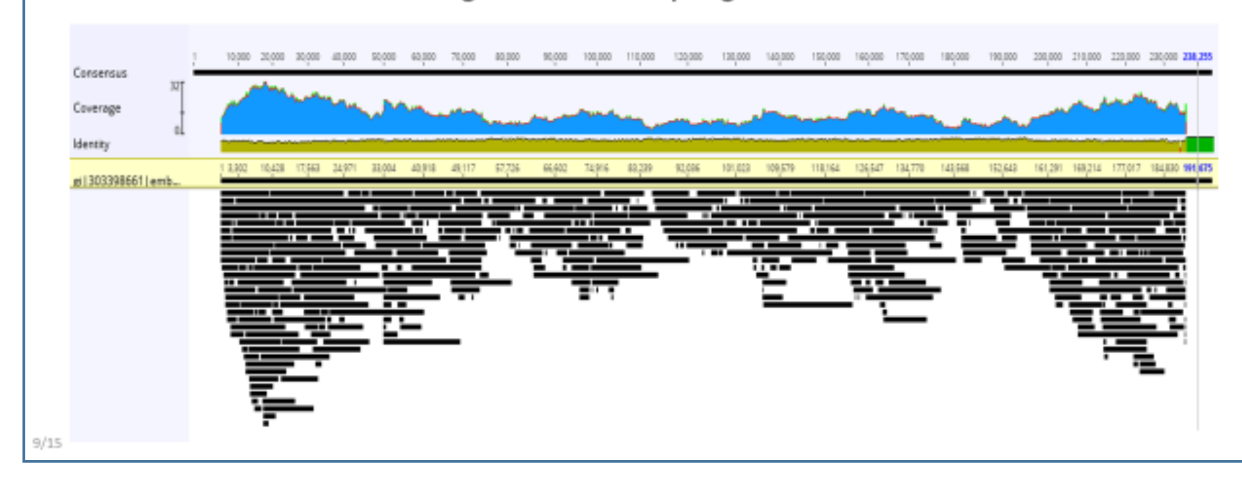




Full Genome Sequencing of ASFV on MinION in Ukraine

Key Accomplishments as of August 2019:

- Library preparation and full genome sequencing tested on clinical isolate
- Annotation of full ASFV genomes is in progress

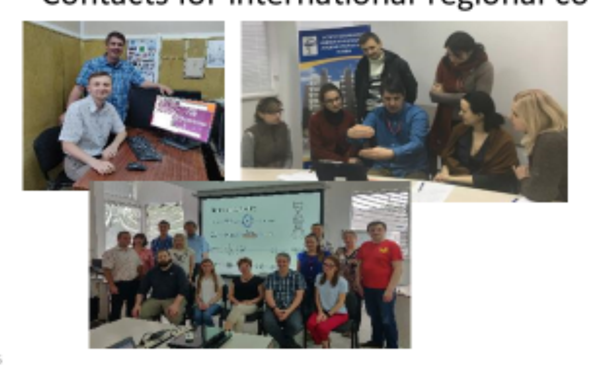


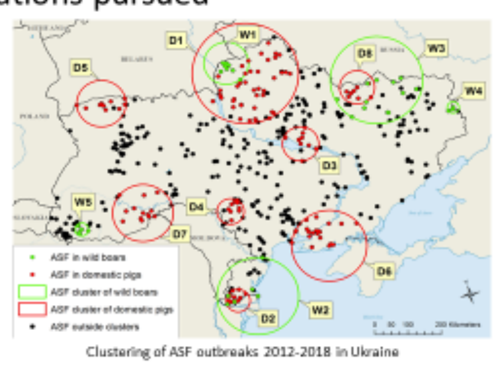
9/15

Bioinformatics, Epidemiological Analyses of ASF Spread

Key Accomplishments as of August 2019:

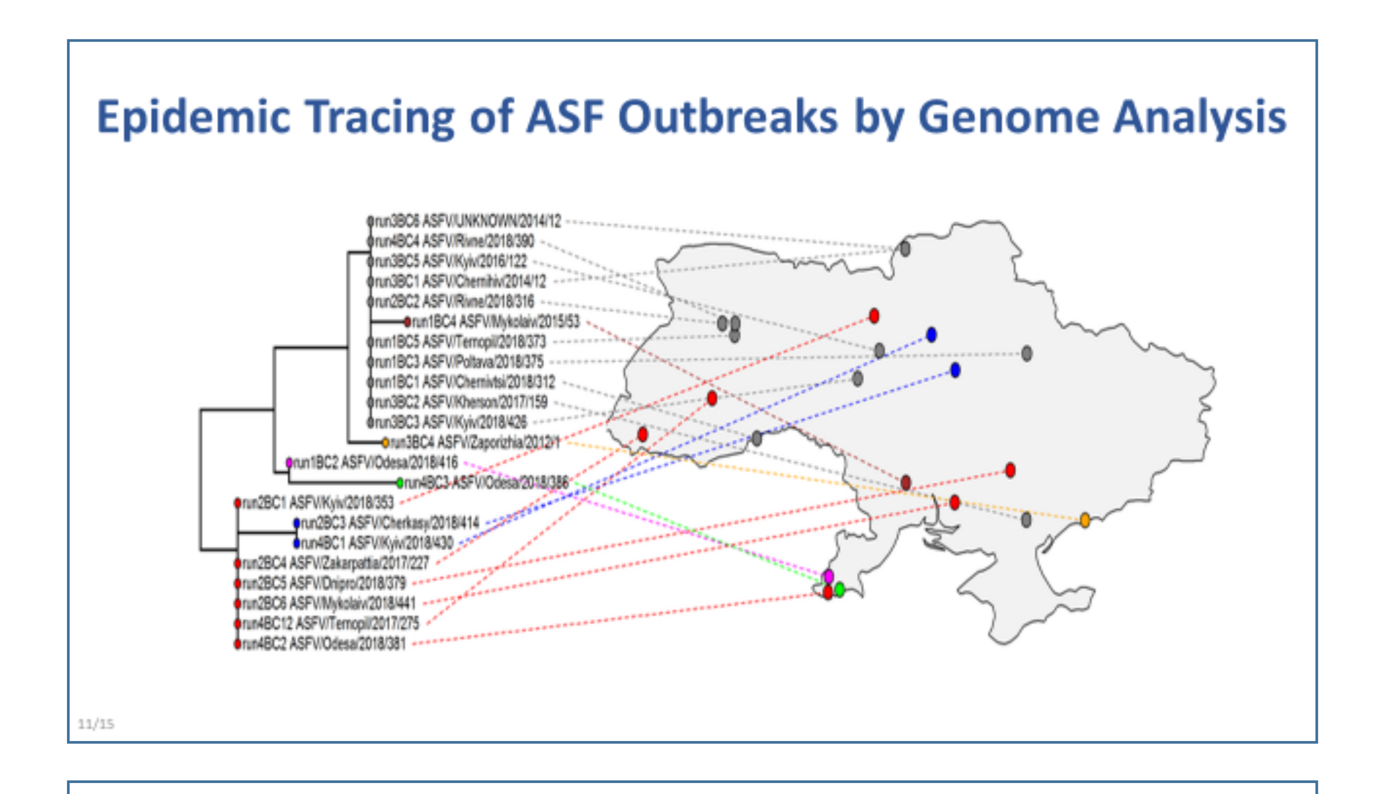
- A server for bioinformatics calculations was installed at IVM (Kyiv, Ukraine)
- Initiated analysis of co-infections (Kharkiv, Ukraine)
- Two workshops conducted with engagement of al Georgian Partners
- Contacts for international regional collaborations pursued





Clustering of ASF outbreaks 2012-2018 in Ukraine

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Threat Reduction Impacts

- Identification of internal and cross-border origins of virulent ASFV genotype ASFV Georgia/2007 lineage outbreaks
- Technical capacity for sequencing ASF and other Especially Dangerous Pathogens (EDPs), by Ukrainian scientists
- Technical capacity for computational (bioinformatics) analysis of ASFV sequences for epidemiological mapping
- Improved communication with institutional and state laboratory stakeholders to improve of ASF control measures
- Integration of Ukrainian scientists and Institutes into the international scientific community
- Adoption of international diagnostic standards

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Publications and Presentations

Publications

- Kovalenko, *et al.* Complete genome sequence of virulent African swine fever virus isolated from a domestic pig in Ukraine, ASM Microbiology Resource Announcements, (*under review*)

Presentations

- 2018 Nanopore Community Meeting (November, San Francisco)
- 2019 American Society of Virology meeting (July, Minneapolis)
- 2019 Regional One Health Research Symposium (May, Kyiv)

Posters

- 2019 ASM Biothreats (February, Washington DC)

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Challenges that have been overcome

- Sequencing capacity in Ukraine began at "0" and achieved "full virus genome sequencing" and bioinformatics expertise through UP-9 Scientific Advancement and capacity building
- ASFV had never been sequenced before on MinION, and never from a clinical DNA archive sample; protocols were developed for the project to achieve this
- Scientists in different institutions in Ukraine and the region had never collaborated on ASF research; this project's workshops brought them together to analyze patterns of ASF infection, to inform control measures

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Next Steps

- Amplicon sequencing of additional ASFV strains in Ukraine and the region, to build a regional epidemic tracing map of ASF spread
- Analysis and annotation of full length ASFV genomes to discover genetic factors that may affect transmission
- Workshops on epidemiology, genome sequencing, and co-infections with Ukrainian and regional researchers

14/15

Acknowledgements

Domestic partners: Mykola Sushko, etc.

International partners: Eric Bortz, etc.

(5) UP-9 presentation at the Regional One Health Science Symposium 2019, Kyiv, Ukraine

- **Abstract 1**

Bioinformatics Efforts as a Part of UP-9 Project Implementation within Cooperative Biological Engagement Program in Ukraine

Sapachova M.[1], Sushko M.[1], Bezimennyi M.[2], Kovalenko G.[2], Usachenko N.[1], Rudova N.[3], Solodiankin O.[3], Tarasov O.[2], Ukhovskyi V.[2], Gerilovych A.[3], Mezhenskyi A.[1], Drown D.[4], Bortz E.[2,5], Dubchak I.[6]
[1]*State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise;*
[2]*Institute of Veterinary Medicine of the NAAS of Ukraine;*
[3]*NSC Institute of Experimental and Clinical Veterinary Medicine of the NAAS of Ukraine;*
[4]*University of Alaska Fairbanks, USA;*
[5]*University of Alaska Anchorage, USA;*
[6]*Lawrence Berkeley National Laboratory, USA*

Introduction. Recent technological advances in genomics have opened unprecedented opportunities for fast sequencing and analysis of pathogenic species in disease outbreaks. To meet the challenge of timely interpretation of structure, function and meaning of new viral sequences we included regular bioinformatics training as a part of UP-9 project “The spread of African swine fever virus (ASFV) in domestic pigs and wild boar in Ukraine – Building capacity for insight into the transmission of ASFV through characterization of virus isolates by genome sequencing and phylogenetic analysis”.
Methods. We developed a training program in bioinformatics and in 2018-2019 organized three weeklong ASFV Genomics & Scientific Advancement workshops in Kyiv, Ukraine. These face-to-face workshops led by Subject Matter Experts (SMEs) were a key event in advancement of computational expertise among Ukrainian scientists participating in UP-9. The goal was to familiarize the participants with the basics of major bioinformatics methods and their practical implementation in various tools.
Results. The course was balanced between lectures and tutorial sessions with hands-on exercises and demonstrations using publicly available on-line bioinformatics resources for viral sequence assembly and genome analysis. During the workshop SMEs presented and drilled participants in the following five modules: 1) Principles of sequence alignment, practical approaches, and NCBI BLAST programs; 2) Global sequence alignment and the VISTA family of tools for comparative genomics; 3) Review of viral genome resources including NCBI Viral Genome Resource and Virus Variation Resource, NIAID/JCVI, and JGI databases; 4) Constructing phylogenetic trees, methods and use cases; 5) Scenarios for the genomic analysis – demonstrating NCBI programs for

alignment and annotation, IQ-tree for phylogenomic inferences and GeneMark for gene prediction.
Each module was presented as a lecture followed by practical exercises, a discussion of the results, and planning of the next steps. Widely used by the scientific community, genomic databases such as GenBank, EMBL Nucleotide Sequence Database, DDBJ-DNA Data Bank of Japan, and PDB (Brookhaven Protein DataBank) were discussed. The basic types of database searches for the nucleotide sequences of ASFV were investigated. Other types of databases (guided, automatic, industrial, integrated), tools for searching similarities in databases (BLAST, PSI-BLAST, FASTA, BLAT), tools for pairwise and multiple alignments and creation of phylogenetic trees were presented and studied as well.
Conclusions. As a result of our emphasis on bioinformatics training the program participants have been able to successfully annotate and analyze the sequences of the Ukrainian strains of ASFV sequenced as a part of the UP-9 project.

- **Abstract 2**

Nanopore (MinION) Sequencing of African Swine Fever Virus in Ukraine

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Introduction. African swine fever (ASF) is a highly contagious hemorrhagic disease that causes high mortality in domestic swine and wild boar, and significant losses for the pork industry.
Methods. Within the state ASF surveillance activity of Ukraine, 4944 samples from wild boar and 21,381 from domestic animals were studied using polymerase chain reaction (PCR). Nanopore (MinION) sequencing technology and bioinformatics were used for African swine fever virus (ASFV) in-depth studies.

Results. During the PCR diagnostics, 163 suspected cases were confirmed positive for ASFV in 2017 and 123 in 2018. Locations of ASF outbreaks were analyzed using geographic information systems (GIS), suggesting a north-to-south and east-to-west trends of spread from epicenters. ASF outbreaks have appeared in both wild boar and domestic swine and are controlled by implementation of ASF control zones around infected farms. However, the rapid spread and persistence of the disease suggests that the genetics of the causative African swine fever virus (ASFV), and the underlying epidemiology of ASF, are not well understood.

To better understand ASFV genetics, we sequenced ASFV DNA using nanopore (MinION) sequencing technology and bioinformatics. This in-depth study of ASFV in Ukraine was initiated under the UP-9 research project, supported by the US Defense Threat Reduction Agency's Biological Threat Reduction Program. Two nanopore sequencing library preparation protocols were developed for PCR amplicons and whole genome sequencing. For PCR amplicon analysis, 13 loci (22 genes) in one novel strain isolated from domestic pigs in Ukraine (ASFV/Zakarpattia/2017/243), were prepared using a LSK-108 library and sequenced on MinION. Whole genome sequencing using the rapid library preparation was also completed on three strains, two from domestic pig (ASFV/Chernihiv/2014/7 and ASFV/Mykolaiv/2015/53), and one from wild boar (ASFV/Kiliiskyi/2018/398). Despite the high abundance of reads matching the host, 30-60 times coverage of the entire ASFV genome was recovered with only eight hours of sequencing. All strains were closely related to the virulent ASFV/Georgia/2007, p72-genotype II lineage, providing a genomic look into the ASF outbreaks in Ukraine.

Conclusions. Protocols developed for analysis of genomic signatures in ASFV strains, as well as improvement of reporting among the project participants and collaborators, will provide critical information for ASF epidemic tracing and outbreak control.

(6) UP-9 Abstract submitted for 2020 ROHRS (postponed to 2021)

- **Abstract**

  Nanopore (MinION) Sequencing of Porcine Circovirus type 2 in Ukraine

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Third-generation nanopore sequencing using MinION technology offers an accessible tool to rapidly prepare virus DNA and analyze sequence data, to identify circulating strains of viruses. We performed nanopore sequencing of porcine circovirus 2 (PCV2) DNA isolated from archival samples in order to understand their genetics and phylogeographic origins.

The capsid gene of 12 PCV2 DNA samples from Ukraine was amplified by polymerase chain reaction (PCR) using specific primers designed for Ukrainian PCV2. PCR amplicons were barcoded for library preparation, and sequenced on a MinION device. Raw nanopore read data was basecalled and quality controlled to generate reads for genome assembly. PCV2 capsid sequences were analyzed using the nanopolish program and assembled to a PCV2 reference strain from Austria, 2003 (AY424401), to generate consensus sequences for phylogenetics analysis. Eleven (11) of the 12 sequences had a high quality read depth >100 reads per nucleotide. Consensus sequences for all 12 sequences covered the capsid gene of the PCV2 (nucleotides 960-1780 in the PCV2 genome). Capsid gene sequences from reference PCV2 subtypes were downloaded from NCBI GenBank. Multiple sequence alignment (MSA) and a phylogenetic tree were constructed using MAFFT (bootstraps: 1000). By this method, a second phylogenetic tree was generated that included the 12 Ukrainian PCV2 sequences from MinION sequencing. Most (11/12) of the Ukraine PCV2 were subtype B, and belonged to a large clade that included PCV2 isolates from Austria, Slovakia, Hungary, Romania, and China. One strain was provisionally identified in the novel subtype F from China. These results suggest that while most PCV2 subtypes in domestic swine in Ukraine are transmitted in a regional context (a Central/East European group), long distance transfer of virus across Eurasia or from China may be possible. These results highlight modes for spread of swine diseases including PCV2, but potentially also African swine fever, swine influenza, and other co-infecting/concurrently infecting swine pathogen.